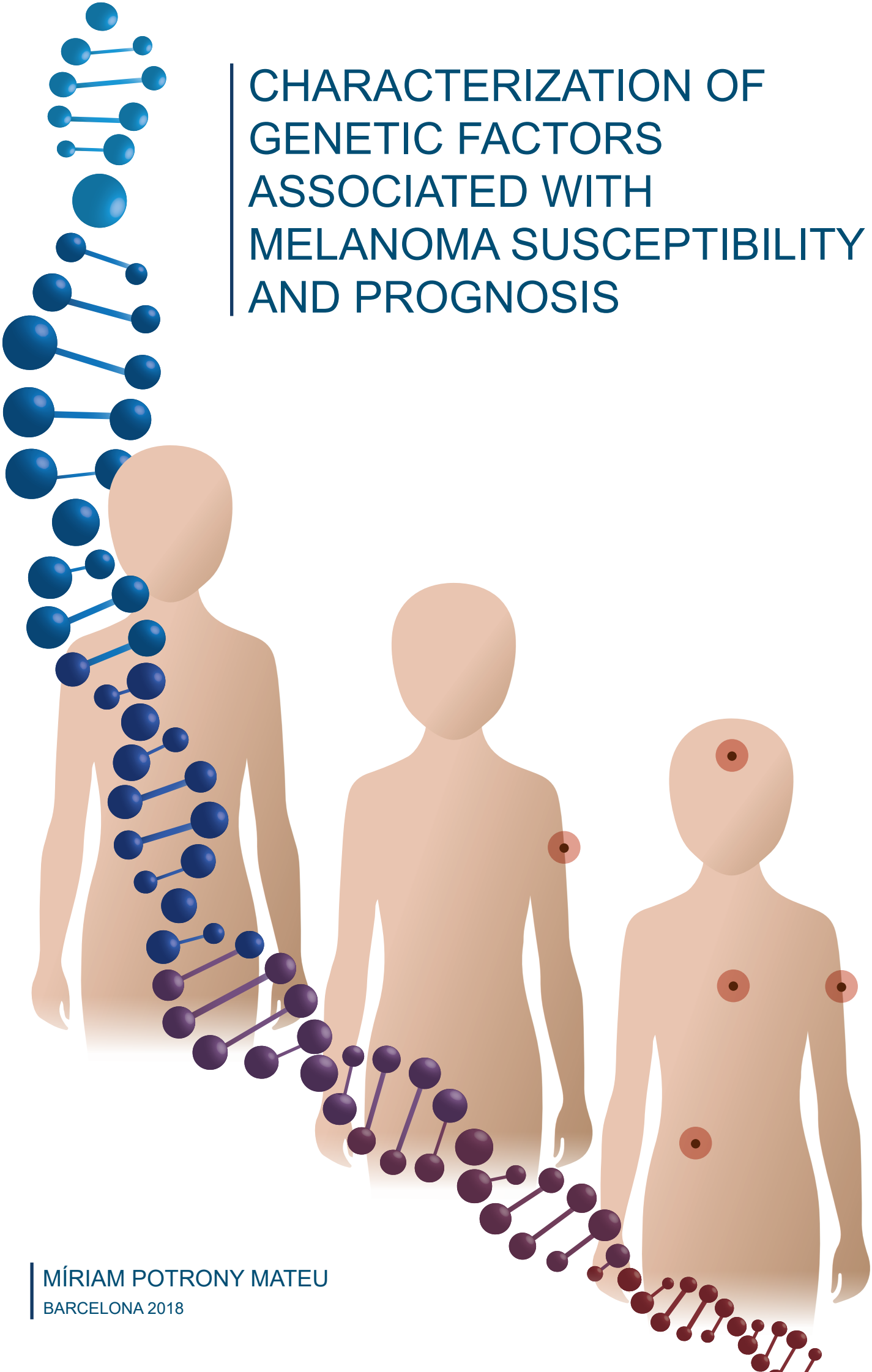
UNIVERSITAT DE BARCELONA

Characterization of genetic factors associated with melanoma susceptibility and prognosis

Míriam Potrony Mateu

CHARACTERIZATION OF GENETIC FACTORS ASSOCIATED WITH MELANOMA SUSCEPTIBILITY AND PROGNOSIS



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BARCELONA 2018

Doctoral Thesis

Faculty of Biology

Department of Genetics, Microbiology and Statistics

Doctoral Program in Genetics

CHARACTERIZATION OF GENETIC FACTORS ASSOCIATED WITH MELANOMA SUSCEPTIBILITY AND PROGNOSIS

Thesis presented by **Míriam Potrony Mateu** to qualify for the degree of Doctor by the University of Barcelona

The thesis was performed at the Melanoma Unit of IDIBAPS-Hospital Clínic of Barcelona, under the supervision of Dr. Susana Puig Sardà and tutorized by Dr. Susana Balcells Comas

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Barcelona, 2018

Acknowledgments/Agraïments

Una tesi és resultat del treball i l'esforç de molts anys i de la implicació directa i indirecta de moltes persones. Aquesta no hauria estat possible sense l'esforç i dedicació de tots els membres de la Unitat de Melanoma que durant més de 20 anys han estat visitant els pacients i les seves famílies, recollint totes les seves dades clíniques i mostres biològiques. Gràcies també als pacients per la seva col·laboració i la seva participació i interès en tots els projectes. I gràcies a tots per deixar-me participar en tots els projectes que han suposat l'eix vertebral de la tesi, però també en tots els altres treballs que hem fet durant aquests anys. És un autèntic plaer poder treballar amb tots vosaltres!

En primer lloc voldria donar les gràcies a les persones que van fer possible que pogués formar part d'aquest grup. Al Dr. Josep Sánchez per recomanar-me anar a parlar amb la Dra. Teresa Estrach per buscar un grup on fer pràctiques, i a ella que em va posar en contacte amb la Dra. Susana Puig. A tu Susana, gràcies per donar-me la oportunitat de formar part del vostre grup, gràcies per encomanar-me el teu entusiasme per la recerca i el melanoma, i moltes gràcies per tota la formació acadèmica i personal que he rebut durant aquests anys al vostre costat!

En segon lloc agrair a també a tots els companys de laboratori que he tingut durant aquests anys. Al Joan Anton que va ser la persona que em va començar a formar en el camp de la recerca en melanoma i que és va convertir en un referent. A la Cèlia per introduir-me en el món de la genètica clínica. A l'Estefi per ensenyar-me les principals tècniques de laboratori. A la Gemma, amb qui des de pràcticament el principi vam estar compartint aquesta etapa formativa, aprenent juntes compartint temps, material, espai, recursos, converses i tots els moments viscuts. També agrair a les incorporacions més recents, a la Judit i a la Neus. Noies, gràcies per formar part del dia a dia. És un gust venir a treballar cada dia amb vosaltres! Pol, a tu també moltes gràcies. Ara comences una nova etapa, però durant el temps que vas estar amb nosaltres al laboratori sempre ens encomanaves el teu bon humor i passàvem molts bons moments gràcies al teu enginy. Marta, tu tot i no formar part directament del grup, també formes part del nostre dia a dia (i més tenint-te al costat), també gràcies per tot el que fas per nosaltres. Finalment, agrair a totes les persones que han passat de pràctiques o estada durant aquest temps pel laboratori: l'Anna, l'Aida (amb qui tants moments bons vam viure al laboratori però també fora d'ell!), la Ilona, la Marta, l'Ari, la Mònica, la Maria, la Nerea i el Tariq; amb alguns de vosaltres vaig poder estar més temps al vostre costat ajudant-vos en la vostra formació i cal dir que aquest és un procés bidireccional en el qual jo he après tant o més que vosaltres.

I would also like to thank Lisa for the welcoming received when I was doing my lab visit at the University of Utah. Thanks to Craig, Jim, Jeff, Jill, and everyone there! Thanks for your counsels and the knowledge you pass on me. Thanks also for the good time spent together and thanks for making my stay there much easier!

Però això va molt més enllà de l'etapa professional viscuda amb tots vosaltres. He de donar les gràcies al papa i la mama que des del moment que vaig néixer han estat al meu

costat. Gràcies per donar-me la oportunitat de perseguir els meus somnis, gràcies per encoratjar-me sempre a seguir endavant i fer-me costat en les decisions que han suposat un gran canvi a la meva vida (i de retruc a la vostra).

A la Noemí, gràcies per estar allà quan ho he necessitat. Des de petitones ho vam estar compartint tot i quan vaig anar a la Universitat ens vam separar físicament, però això no ha canviat el fet que quan ens necessitem l'una a l'altra, estem allí! Gràcies també per tots els dissenys de les portades dels treballs que m'has preparat al llarg dels anys i també la d'aquesta tesi (Albert, perdona per haver-te-la robat unes hores).

També donar les gràcies al padrí i la padrina per preocupar-se de com em va en el món de la recerca. No puc imaginar-me la il·lusió que deu fer tenir una néta presentant una tesi doctoral. Però com ells em diuen, això no ho sabré fins que m'hi trobi jo!

Aquest dia sé que hauria fet particularment il·lusió a la iaia. Vostè ja no hi és per viure'l en persona, però en aquest moments recordo molt les nostres converses sobre el doctorat, quan ni tan sols havia començat a fer les pràctiques de la carrera. I la veritat iaia, és que ha estat una etapa molt millor del que m'imaginava!

Som una família petita així que em puc permetre el luxe de nombrar a la resta de persones que heu estat al meu costat durant aquest temps, la padrineta, el tiet i el padrí. Gràcies per estar al meu costat. I Laia, no sé que faràs quan siguis gran, però tal com vaig fer jo t'aconsello que treballis molt i lluitis pels teus somnis! Val molt la pena!

Arribar a aquest moment també ha estat possible gràcies als professors d'Institut i de la Universitat que van saber captar el meu interès per la ciència i la genètica del càncer. Gràcies a tots vosaltres avui estic aquí. També agrair en particular a la meva tutora, la Dra. Susana Balcells per donar-me suport durant aquest procés.

I finalment no em puc deixar tots els meus amics fora de l'àmbit laboral. Als amics de les meves terres que encara seguiu allà; ara ens veiem menys però quan ho fem es com si no hagués passat el temps! També a les Trempolines, gràcies per tots els moments viscuts i pel suport que ens hem anat donant durant aquests anys. I finalment agrair a l'Albert, que en l'últim any s'ha convertit en una persona molt important de la meva vida, gràcies per estar al meu costat!

Per acabar només donar GRÀCIES DE NOU a totes aquelles persones tant de l'àmbit personal com professional que heu estat al meu costat tot aquest temps!!!

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ABBREVIATIONS

AJCC: American Joint Committee on Cancer

ALM: Acral lentiginous melanoma

CI: Confidence interval

DNA: Desoxiribonucleic acid

eQTL: expression quantitative trait locus

GWAS: Genome-wide association studies

HR: Hazard ratio

LDH: Lactate dehydrogenase

LMM: Lentigo maligna melanoma

MAPK: Mitogen-activated protein kinase

MPM: Multiple primary melanomas

NGS: Next-generation sequencing

NM: Nodular melanoma

OR: Odds ratio

PR: Prevalence ratio

RCC: Renal cell carcinoma

RHC: Red hair color

RNA sequencing: RNA-seq

RNA: Ribonucleic acid

RR: Relative risk

SLN: Sentinel lymph node

SNP: Single nucleotide polymorphism

SSM: Superficial spreading melanoma

TIL: Tumor-infiltrating lymphocytes

UVR: Ultraviolet radiation

WES: Whole-exome sequencing

WGS: Whole-genome sequencing

Genes and proteins

α-MSH: Alpha melanocyte-stimulating hormone

ACD: ACD, Shelterin Complex Subunit And Telomerase Recruitment Factor

BAP1: BRCA1 (Breast cancer 1) associated protein 1

BRAF: B-Raf Proto-Oncogene, Serine/Threonine Kinase

CD5: CD5 Molecule

CDK4: cyclin-dependent kinase 4

CDKN2A: cyclin-dependent kinase inhibitor 2A

CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

CXCL1: C-X-C Motif Chemokine Ligand 1

DLG2: Discs Large MAGUK Scaffold Protein 2

FZD4: Frizzled Class Receptor 4

GOLM1: Golgi Membrane Protein 1

HLA: Human leukocyte antigen, also known as MHC, major histocompatibility complex antigen

IDO: indoleamine-pyrrole-2,3-dioxygenase-1,2

IL10: Interleukin 10

IL8: interleukin 8

LAG3: lymphocyte-activation gene 3, CD223

MC1R: Melanocortin 1 Receptor

MITF: Microphthalmia-associated transcription factor

NRAS: NRAS Proto-Oncogene, GTPase

PARP1: Poly ADP-Ribose Polymerase 1

PD-1: Programmed death-1

PD-L1: Programmed Death-ligand 1

POT1: Protection of telomeres 1

PRSS23: Protease, Serine 23

RB: retinoblastoma protein

TERF2IP: TERF2 (Telomeric Repeat Binding Factor 2) Interacting Protein

TERT: Telomerase reverse transcriptase

TIM-3: T-cell immunoglobulin domain and mucin domain 3

TMEM135: Transmembrane Protein 135

Treg: regulatory T cell

VDR: Vitamin D Receptor

α-MSH: Alpha melanocyte-stimulating hormone

INTRODUCTION

MELANOMA

Melanoma is the type of skin cancer arising from the melanocytes. Melanocytes are cells derived from the neural crest and are located mainly in the skin (epidermal basal layer and hair follicles), but also can be found in the eye, the brain, and mucosa. Mature melanocytes are oval or fusiform, dendritic cells, responsible for melanin synthesis (melanogenesis). Two major types of melanin are produced: eumelanin (brown-black pigment, high photoprotecting properties) and pheomelanin (yellow-red, non-photoprotecting and carcinogenic). Melanin is encapsulated within the melanosomes that are transferred through the melanocyte dendrites to the surrounding keratinocytes. Melanosomes are then placed above the keratinocyte nucleus and become a protective barrier against ultraviolet radiation (UVR) (**Figure 1**). The ratio eumelanin/total melanin is responsible for skin color determination.[1]

In the epidermal basal layer the melanocyte:keratinocyte ratio is 1:10 and each melanocyte establishes relationships with 30-40 keratinocytes.[1] This balance is maintained through regulated induction of melanocyte division and is only disturbed during transformation into a nevus (benign melanocytic tumor) or a melanoma (malignant melanocytic tumor). In order to proliferate, melanocytes need to decouple from the basement membrane and from the keratinocytes, retract their dendrites, divide, and migrate along the basement membrane before they finally recouple to the matrix and to keratinocytes to form another epidermal melanin unit. Keratinocytes control melanocyte growth and expression of cell surface receptors. During tumor development, melanoma cells escape from control by keratinocytes.[2] Human melanocyte proliferation requires the cross-talking of several signaling pathways including the MAPK (mitogen-activated protein kinase) and α-MSH/MC1R/MITF. Molecular deregulation of those pathways can lead to the abnormal melanocyte proliferation.[3]

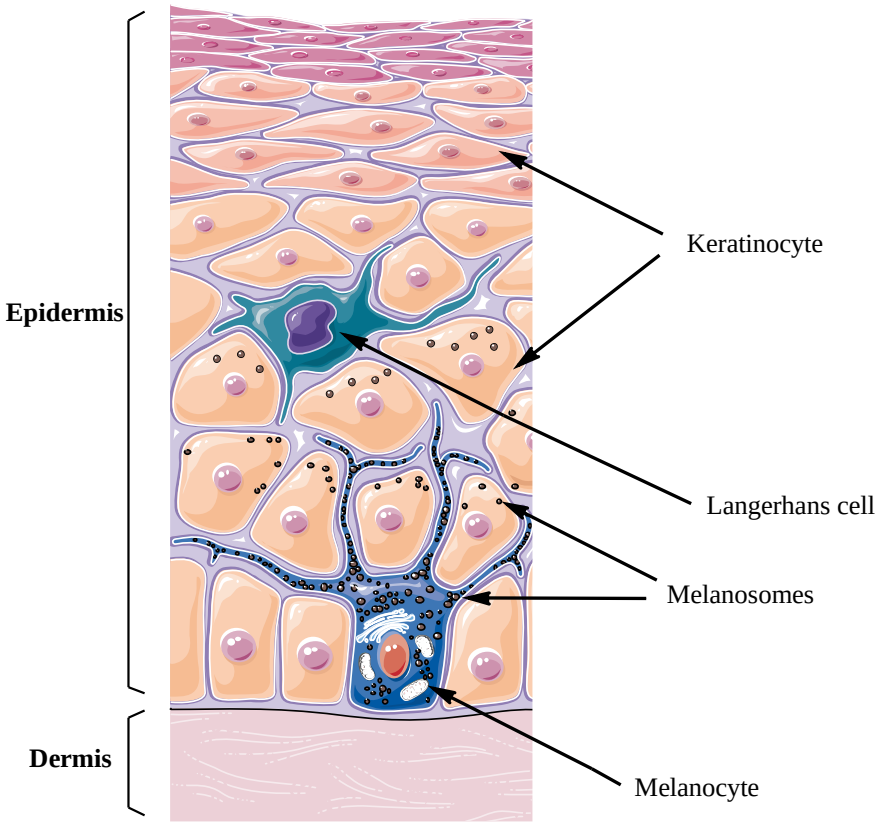

Figure 1. Schema of melanocyte location in the skin

MELANOMA SUSCEPTIBILITY

Each year >250,000 melanoma cases are diagnosed worldwide, with an age-standardized rate per 100,000 person-year of 4.8 in males and 3.5 in females.[4] The highest incidence is reported in Australia and New Zeeland (>30), followed by Northern America and Northern Europe (~14), lower in Southern Europe (~8), and the lowest incidence is reported in Asia and Africa (~0.5-1).[5] Melanoma incidence has increased by 39% from 2006 to 2016 worldwide. The 15% increment was due to a change in the population age-structure, 12% to population growth, and 11% to a change in age-specific incidence rates.[4] In Catalonia, more than 700 new cases are diagnosed per year and the incidence is also continually increasing. We detected an increase of 1.9 points of age-standardized rate for the European population in eight years, for all melanomas including *in situ*, and a 1 point increase for invasive melanomas.[6]

Melanoma etiology is complex and heterogeneous, involving environmental and intrinsic factors. The main environmental risk factor for melanoma is UVR exposure.

Both the total sun exposure and sunburn history increase melanoma risk (**Table 1**).[7] UVR causes DNA (Desoxiribunoucleic acid) damage through the formation of pyrimidine dimers, photoproducts, gene mutations, oxidative stress, inflammation, and immunosuppression, favoring the carcinogenic process.[8] UVR has been widely demonstrated to be implicated in the development of nevi and melanoma. UVR can induce clinical changes (increased pigmentation, scaling and erythema). The use of sunscreen can prevent part of the UVR effects on nevi.[9] Furthermore, a 10-year follow-up study showed that the daily use of sunscreen reduces the melanoma detection rate, suggesting that regular sunscreen use may prevent melanoma development.[10]

Clinical and host characteristics

Phenotypic characteristics such as red or blond hair, blue or green eyes, fair skin with low tanning ability, freckles, multiple melanocytic nevi or presence of atypical nevi are associated with an increased risk to develop melanoma.[11, 12] Having a high number of nevi or dysplastic nevi is the major phenotypic risk factor (**Table 1**). However, the risk of any particular nevus becoming melanoma is low: for a 20-year-old individual, the lifetime risk of any selected nevus transforming into melanoma by age of 80 is approximately 0.03% (1 in 3,164) for men and 0.009% (1 in 10,800) for women.[13] Personal history of melanoma also increases from 5% to 8% the risk of developing a second melanoma.[14, 15] Finally, family history of melanoma has been widely associated with an increased melanoma risk (**Table 1**).[12, 16]

Genetic susceptibility factors

Melanoma is the tumor with the highest heritability (58%).[17] Sporadic melanoma accounts for 90% of cases and 5-10% of those individuals develop multiple primary melanomas (MPM). On the other hand, 10% of melanoma cases occur in a familial context. In families with melanoma aggregation, melanoma susceptibility follows an autosomal dominant inheritance pattern with incomplete penetrance.

UVR	**RR**	**95% CI**	**Reference**
Total sun exposure	1.34	1.02-1.77	7
Sunburns	2.03	1.73-2.37	7
Phenotypic features	**RR**	**95% CI**	**Reference**
Red hair vs. dark	3.64	2.56-5.37	12
Blond hair vs. dark	1.62	1.11- 2.34	12
Blue eyes vs. dark	1.47	1.28-1.69	12
Green eyes vs. dark	1.61	1.06-2.45	12
Fair skin	2.06	1.68-2.52	12
Low tanning ability	2.09	1.67-2.58	12
High density of freckles	2.10	1.80-2.45	12
≥100 nevi	6.89	4.63-10.25	11
≥5 atypical nevi	6.36	3.80-10.33	11
Familial history	1.74	1.41-2.14	12
Genetic factors	**OR**	**95% CI**	**Reference**
CDKN2A mutations*	86*	30-132*	27
MC1R one variant	1.42 to 2.45[§]	-	48
MC1R two RHC variants	5.02	2.88-8.94	50
MITF p.Glu318Lys	2.19 to 8.37[†]	-	54-55

Table 1. Summary of melanoma risk according to UVR indicators, phenotypic features and high/medium-risk genetic factors

RR: Relative risk; 95% CI: 95% confidence interval; OR: Odds ratio

RHC: red hair color variants (included in the analysis: c.252 C>A p.Asp84Glu, c.425 G>A p.Arg142His, c.451 T>C p.Arg151Cys, c.478 C>T p.Arg160Trp and c.880 G>C p.Asp294His)

*Mutation penetrance estimation.

[§]OR differences are observed depending on the MC1R variant.

[†]OR differences are observed depending on family and personal history of melanoma.

Genetic susceptibility can be explained due to the inheritance of low, medium or high-risk variants, or a combination of them (reviewed in **Annex 1**).[18] The environmental factor effect may be higher when low-risk variants are inherited while less necessary when high-risk variants are inherited. Familial melanoma cases are more likely to be explained by the inheritance of high-risk or accumulation of medium-risk variants, while sporadic cases are more likely to be explained by the accumulation of low or medium-risk variants (**Figure 2**). However, as UVR is the main environmental risk factor for melanoma, families with low- to medium-risk variants living in areas with an increased UVR could have more melanoma cases.[19]

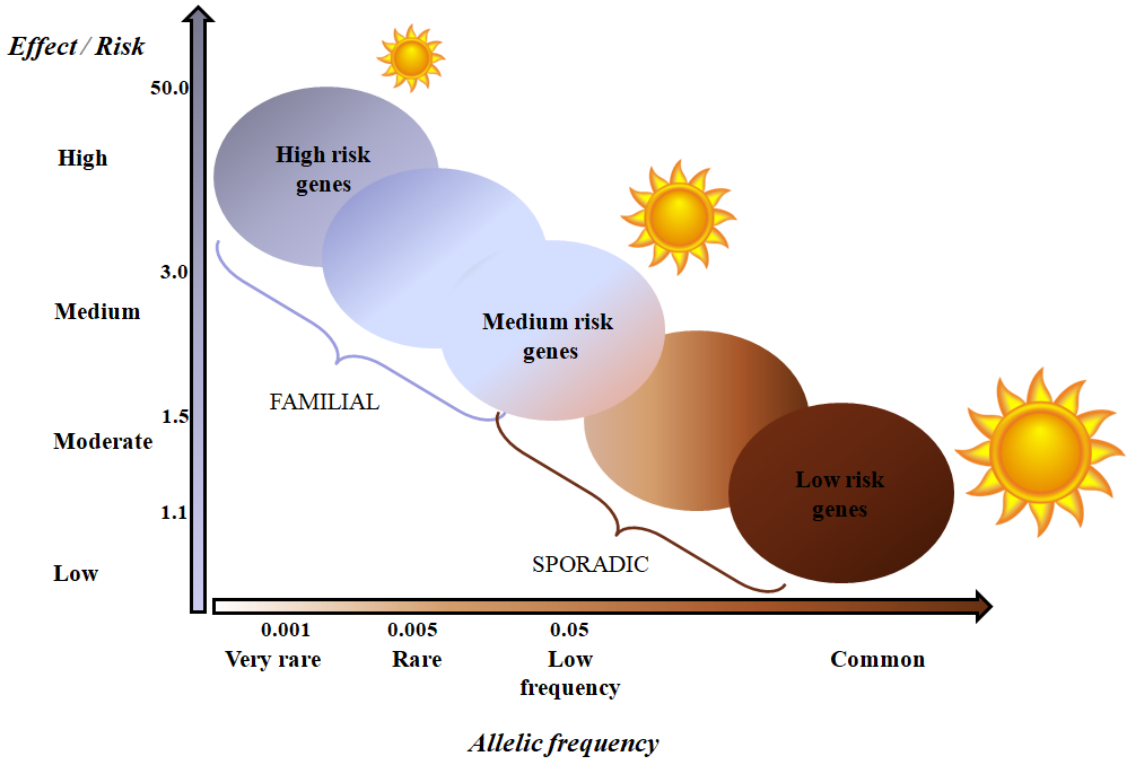

Figure 2. Genetic inheritance model for familial and sporadic melanoma.
Adapted from Manolio et al. 2009.[20]

High-risk genes

Melanoma high-risk genes are defined as genes that when mutated in an individual confer a high-risk of developing melanoma and are usually associated with multiple melanoma cases within the family. *CDKN2A* (cyclin-dependent kinase inhibitor 2A) was the first gene associated with melanoma susceptibility. Linkage analyses allowed the identification of 9p21 as a familial melanoma locus in 1992.[21] Two years later, the

first germline mutations in *CDKN2A*, located in the 9p21 locus, were reported in familial melanoma.[22] The *CDKN2A* gene encodes two tumor suppressor proteins p16INK4A and p14ARF via differential splicing and alternative reading frames. The protein p16INK4A, encoded by the alpha transcript (composed by exon 1 alpha, 2 and 3), promotes the arrest of the cell cycle in the G1 phase by inhibiting RB (retinoblastoma protein) phosphorylation through CDK4 (cyclin-dependent kinase 4). The beta transcript (composed by exon 1 beta, 2 and 3) encodes p14ARF and acts through the p53 pathway inducing the cell cycle arrest or favoring apoptosis.[23] Furthermore, both p53 and p16INK4A play an important role in cell damage response and senescence (**Figure 3**, **Annex 1**).[18, 24] To date, *CDKN2A* is the main high-risk gene involved in melanoma susceptibility. Mutations in that gene are found in around 20% of melanoma-prone families (**Figure 4**, **Annex 1**),[18] but the *CDKN2A* mutation frequency can range from 5% to 72% depending on the selection criteria used and the geographical areas.[25, 26] *CDKN2A* mutation penetrance assessment estimated that carriers have an 86-fold increased risk of developing melanoma (**Table 1**).[27] However, melanoma penetrance in *CDKN2A* carriers varies between geographical areas and increases with age. Bishop and colleagues reported that at the age of 50, the melanoma penetrance for carriers was 13% in Europe, 50% in the US and 32% in Australia, while at the age of 80 the penetrance was 58% in Europe, 76% in the US and 91% in Australia.[28]

CDK4 was the second high-risk melanoma susceptibility gene identified, thanks to a candidate gene screening approach of p16INK4A interacting partners.[29, 30] *CDK4* is an oncogene located within the 12q14 chromosomal region and encodes a protein that controls cell cycle progression through the G1 phase. To date, mutations in this gene have been described in 18 melanoma-prone families and in all of them, the mutation affects the same amino acid (Arginine 24).[31, 32] This amino acid is located in the p16INK4A binding domain of the CDK4 protein. Thus, when *CDK4* is mutated, p16INK4A cannot inhibit the CDK4 kinase activity resulting in the progression of the cell cycle. *CDK4* mutation carriers phenotypically behave similarly to p16INK4A mutation carriers.[31] This is consistent with the functional impact that mutations in both genes have at the cellular level, which results in the activation of the same pathway (**Figure 3**, **Annex 1**).[18]

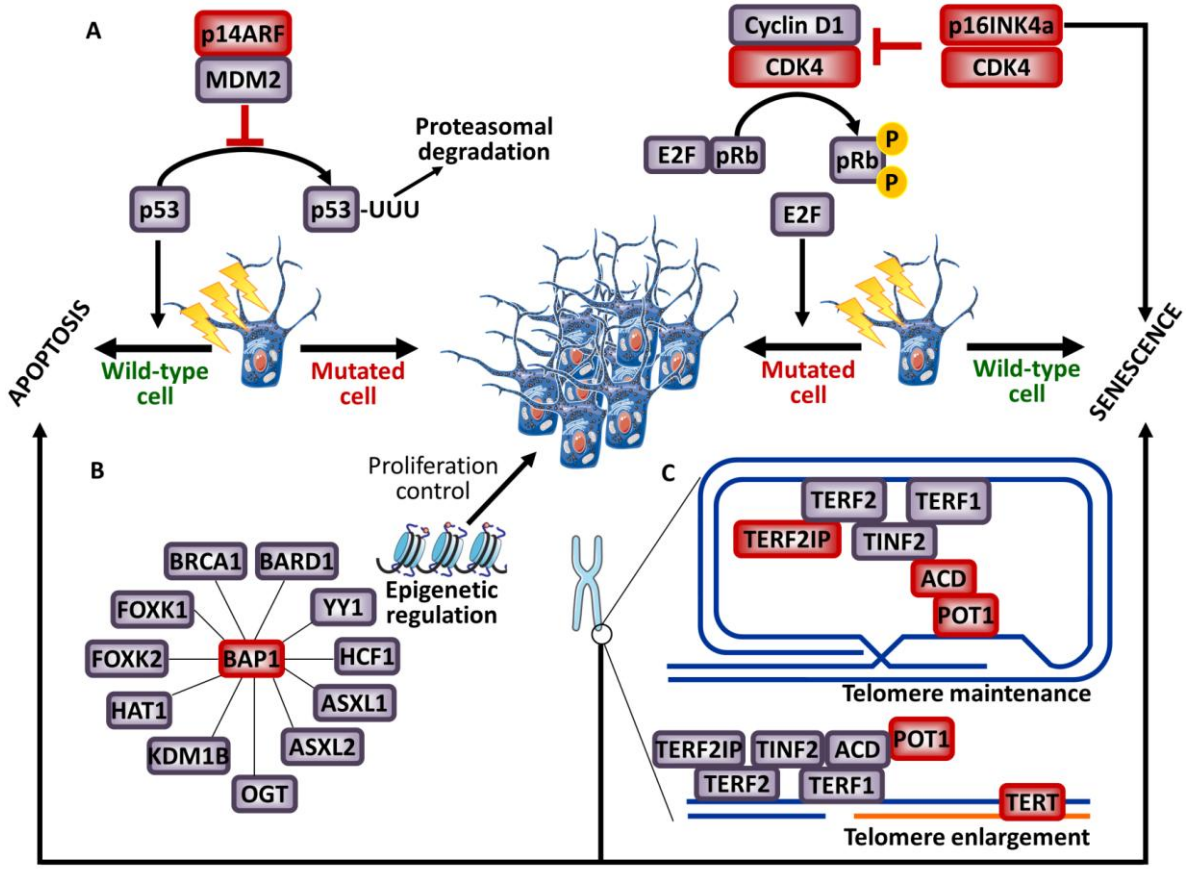

Figure 3. High-risk melanoma genes function

Cell biology functions and pathways of the genes involved in melanoma susceptibility. In red all proteins encoded by known high-risk melanoma susceptibility genes.

a) *CDKN2A* encodes two different proteins p16INK4a and p14ARF. When p16INK4a can perform its function correctly, if a cell is damaged it can induce senescence. However, when *CDKN2A* is mutated, the cyclinD1/CDK4 complex is not inhibited and E2F transcription factor is released being able to innduce a cell cycle progression. If a cell is damaged the release of E2F can favor an aberrant proliferation that can lead to tumor development. In the same way, when *CDK4* is mutated, p16INK4a cannot interact with it and cannot inhibit the release of E2F. p14ARF in front of a damaged cell induces apoptosis through the p53 pathway. If p14ARF is mutated, MDM2 can promote p53 ubiquitination and p53 is degraded in the proteasome. Thus, when a cell is damaged, the lack of activation of p53 can allow that cell to avoid apoptosis. b) BAP1 is involved mainly in epigenetic and gene transcription regulation interacting with multiple partners. When *BAP1* is mutated it cannot properly do its activity and this can lead to a dysregulation of gene transcription that can alter the cell proliferation control. c) Telomeres form a protective structure at the ends of the chromosome (T-loop) that is covered by the shelterin complex (TERF2IP, TERF1, TERF2, TINF2, ACD, and POT1). This complex also mediates the interaction between telomeres and the telomerase (TERT). POT1 binding to the single-stranded DNA overhang prevents access of TERT to telomeres. When POT1 is unbound, the telomerase is able to extend telomeres. Furthermore when the telomeres are unprotected by the shelterin complex, senescence and apoptosis can be induced. Mutations in those genes lead to telomere deregulation and can be involved in the development of a tumor when the cells are damaged.

Source: Potrony et al. 2015,[18] Annex 1.

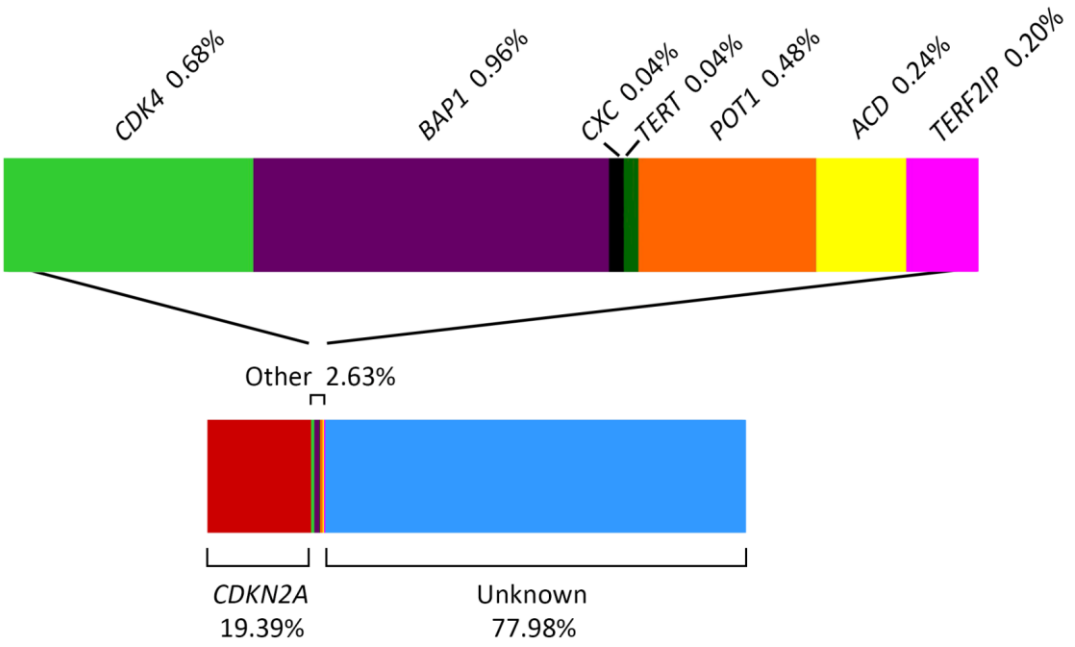

Figure 4. Worldwide prevalence of mutations of high-risk melanoma susceptibility genes in melanoma-prone families

This figure includes the genetic information of 2,511 pedigrees: 487 *CDKN2A*, 17 *CDK4*, 24 *BAP1*, 1 *CXC*, 1 *TERT*, 12 *POT1*, 6 *ACD* and 5 *TERF2IP* mutated pedigrees respectively, and 1,958 families with an unknown mutation. Source: Potrony et al. 2015,[18] Annex 1.

BAP1 (BRCA1 [Breast cancer 1] associated protein 1) was initially identified as a tumor suppressor gene playing a role in melanoma by exome capture coupled with massively parallel sequencing of uveal melanomas.[33] *BAP1* germline mutations have been associated with a cancer syndrome characterized by the presence of broad tumor types: cutaneous melanoma, uveal melanoma, mesothelioma, renal cell carcinoma, atypical Spitz tumors, atypical intradermal tumors (reviewed by Carbone and colleagues[34]) and multiple basal cell carcinomas.[35, 36] However, the whole tumor spectrum associated with germline *BAP1* mutations is still unknown. *BAP1* is located in the chromosomal region 3p21 and encodes a protein that plays a tumor suppressor role through transcription regulation by chromatin remodeling and the ubiquitin-proteasome system. *BAP1* is a deubiquitylase that participates in multiprotein complexes that regulate key pathways including the cell cycle, cell differentiation, cell death, gluconeogenesis, and the DNA damage response.[34] The frequency of *CDKN2A* wild-type melanoma-prone families with mutations in *BAP1* is not well established, but beyond cutaneous melanoma,

families bearing *BAP1* mutations seem to be enriched by other tumors associated with this syndrome.

Genome-wide copy number variant assessment allowed the identification of a duplicated region on 4q13 segregating with melanoma in one melanoma-prone family,[37] suggesting that some melanoma-prone families seem to carry mutations in private genes. The whole duplicated region contains 10 genes, most of them belonging to a family of α-chemokines, such as *CXCL1* (C-X-C Motif Chemokine Ligand 1) and *IL8* (interleukin 8). Both genes have been shown to stimulate melanoma growth in vitro and in vivo.[37]

The most recently identified melanoma high-risk genes are involved in telomere maintenance. Telomeres consist of tandem nucleotide repeats (TTAGGG)n and are located at the ends of chromosomes. The telomerase enzyme, the shelterin protein complex, and many other accessory proteins are also comprised in the telomeres. They maintain genomic stability and chromosomal integrity by protecting chromosome ends from degradation, end-to-end fusion, and atypical recombination.[38] Telomeres shorten both with age and exposures associated with cancer risk, such as smoking and UVR.[39, 40] Thus, telomere maintenance processes are natural candidates for explaining carcinogenesis. Horn and colleagues identified a germline mutation in the promoter of *TERT* (Telomerase reverse transcriptase) in a melanoma-prone family using multipoint linkage analyses and target-enriched high-throughput sequencing.[41] *TERT* is located in 5p15 and encodes the catalytic subunit of the telomerase, which is the ribonucleoproteic complex that maintains telomere length. Two independent groups identified rare germline variants in *POT1* (Protection of telomeres 1) in 12 *CDKN2A* wild-type melanoma-prone families using next-generation sequencing (NGS).[42, 43] *POT1* is located within the 7q31 chromosomal region and encodes a protein of the telomeric shelterin complex. POT1 plays an important role in telomere maintenance by preventing the inappropriate processing of the exposed chromosome ends, caused by pathways related to DNA damage response, and regulating telomerase function.[44] Furthermore, Aoude and colleagues described germline mutations in melanoma-prone families located in two more genes involved in the shelterin complex, *ACD* (ACD, Shelterin Complex Subunit And Telomerase Recruitment Factor) and *TERF2IP* (TERF2 [Telomeric Repeat Binding Factor 2] Interacting Protein). This study included 510

melanoma-prone families without known mutations and identified 6 families with mutations in *ACD* and 4 families with *TERF2IP* mutations, segregating with melanoma (**Figures 3 and 4**, **Annex 1**).[18, 45]

Although several studies have been performed in an attempt to identify new high-risk melanoma susceptibility genes, we still have missing heritability in >70% of families worldwide. There is a need to identify new melanoma susceptibility genes that explain part of the high missing heritability.

Low- to medium-risk genes

MC1R (Melanocortin 1 Receptor) is considered a medium-risk gene and its role in melanoma susceptibility has been widely studied. *MC1R*, located in 16q24, is one of the master regulator genes in human pigmentation and encodes the α-MSH (alpha melanocyte-stimulating hormone) receptor 1. MC1R activation increases the cAMP level and promotes CREB phosphorylation and MITF transcription (**Figure 5**). This results in the transcription of proliferative and anti-apoptotic genes, as well as genes related to the eumelanin synthesis. *MC1R* is a highly polymorphic gene in the Caucasian population. Variants in *MC1R* have different functional effects, either at the level of α-MSH binding or cAMP signaling, resulting in changes in the ratio between eumelanin and pheomelanin.[46] *MC1R* variants were first associated with fair skin and red hair color in 1995.[47] Further studies and meta-analyses have confirmed this association with skin and hair pigmentation.[48] Independently of their phenotypic effect, *MC1R* variants are also associated with an increased risk of developing melanoma (**Table 1**).[48-50] When the *MC1R* function is hardly compromised, this usually results in the red hair color phenotype (RHC). The most common *MC1R* variants have been classified as r variants (low association with RHC: p.Val60Leu, p.Val92Met, p.Arg163Gln) and R variants (high association with RHC: p.Asp84Glu, p.Arg142His, p.Arg151Cys, p.Ile155Thr, p.Arg160Trp, p.Asp294His).[48, 51] The R variants are those most implicated in melanoma susceptibility. The melanoma risk conferred by R variants varies from two times risk per R allele in the general population to three times risk, in the familial melanoma context. The risk is additive, thus carriers of two R alleles have 4 to 6 times increased risk than individuals without these variants. The r variant p.R163Q is also associated with increased risk of melanoma in high sun exposed geographic

areas[52] and with a subtype of melanoma associated with chronic sun damage, lentigo maligna melanoma.[53]

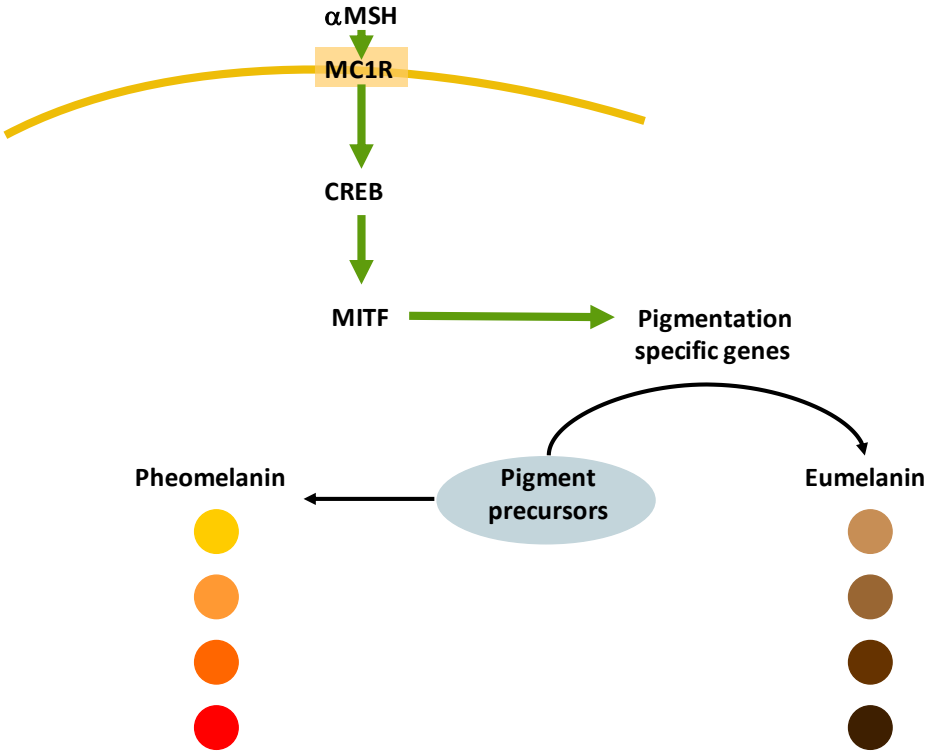

Figure 5. MC1R role in pigmentation

MC1R promotes the expression of pigmentation specific genes needed for the synthesis of eumelanin from pigment precursors. When *MC1R* has variants impairing its function, such as RHC variants, pigmentation specific genes are not synthesized and the pigment precursors form pheomelanin.

MITF (Microphthalmia-associated transcription factor) is also considered a medium-risk gene for melanoma. In fact, *MITF* became the first gene to be identified in melanoma susceptibility using NGS methods.[30] Two independent groups identified the rare functional variant in *MITF* p.Glu318Lys (rs149617956) that increases melanoma risk and also predisposes to renal cell carcinoma (RCC).[54, 55] *MITF* is located in the chromosomal region 3p14 and is a master regulator gene of melanocyte development and differentiation, and it is also associated with melanoma development and progression.[56] *MITF* p.Glu318Lys occurs at a conserved SUMOylation position and this variant decreases the number of SUMO-modified MITF forms. As SUMOylation of MITF represses its transcriptional activity, p.Glu318Lys increases the MITF transcriptional activity and may result in the up-regulation of distinct sets of genes (**Figure 6**). Furthermore, this variant promotes invasive and tumorigenic behaviors in

melanoma and RCC cells and might favor a phenotypic switch of melanoma cells towards a tumor-initiating cell phenotype.[54]

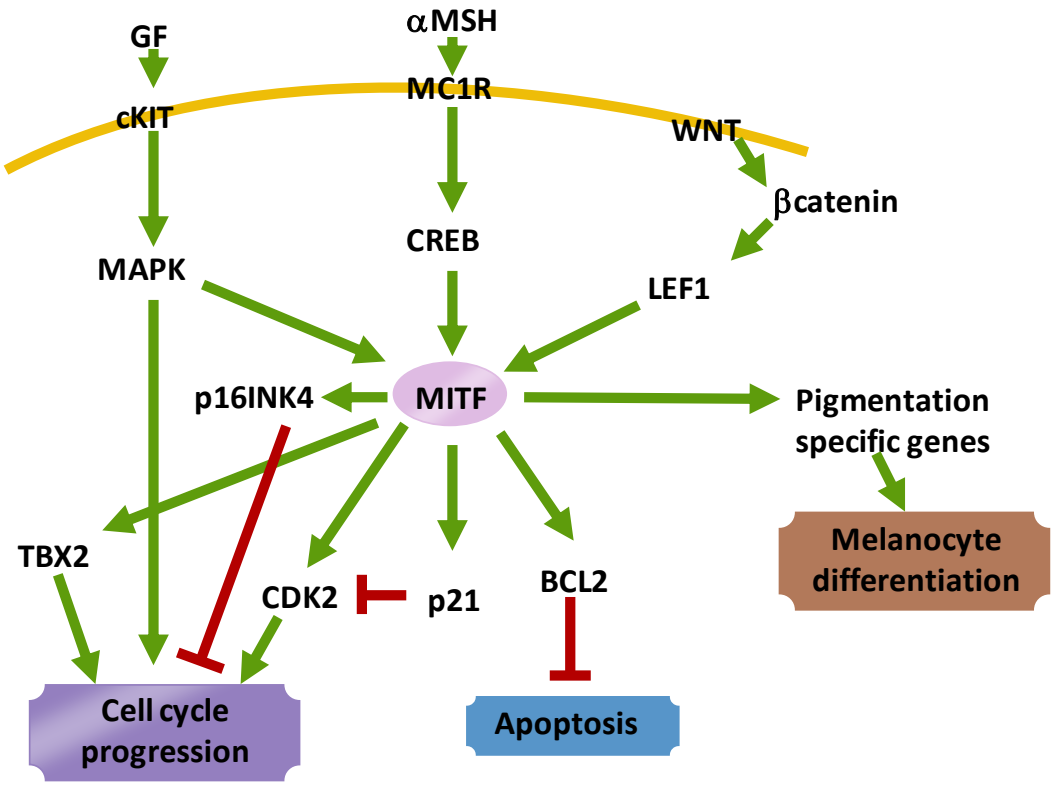

Figure 6. MITF role in the melanocyte

MITF can be activated through different pathways as shown in the image and ends up regulating different processes such as melanocyte differentiation, cell cycle progression, and apoptosis.

Finally, genome-wide association studies (GWAS) and few candidate gene studies allow the identification of common variants associated with melanoma. However, the risk conferred by these common variants is low. Each variant alone does not reach a two-fold increased melanoma risk.[57, 58] These genes are involved in different biological processes including: nevi count and pigmentation,[59-61] immune system,[62, 63] metabolism,[58, 64-66] DNA repair,[63, 67] telomere maintenance, cell proliferation/death, or other mechanisms less understood (**Annex 4**).[58]

Genetic counseling

Genetic counseling is the process of identifying and counseling individuals at increased risk of developing cancer, and distinguishing between those at high-risk (high penetrance genes/families), those at a medium increased risk (multifactorial etiology or

low to medium penetrance alleles), and those at average risk.[68] This information can also be used to assess other at-risk individuals within the family. Genetic counseling is offered to melanoma-prone families and sporadic MPM to better understand the meaning of the disease and genetic susceptibility, the inheritance pattern, the option of genetic testing, the understanding of all the possible results, and the primary and secondary prevention of melanoma as well.[19] The process includes melanoma risk assessment, the possibility of genetic testing, informed consent, the disclosure of test results and psychosocial assessment as in other cancer genetic counseling or assessment.[19, 68]

Leachman and colleagues described a very useful rule to select patients for genetic testing in melanoma according to the melanoma incidence in the general population and the prevalence of mutations in each region. In countries with a low melanoma incidence, such as Southern European countries, the selection criteria for genetic counseling should follow the rule of two: individuals with two primary melanomas and/or families with at least one invasive melanoma and one or more other diagnoses of melanoma and/or pancreatic cancers among first- or second-degree relatives on the same side of the family. While, countries with a moderate to high melanoma incidence, such as the US and Northern European countries, should follow the rule of three: individuals with three or more primary invasive melanomas and families with at least one invasive melanoma and two more cases of melanoma and/or pancreatic cancer among first- or second-degree relatives on the same side of the family. For very high melanoma incidence countries, such as Australia, the rule of four may be suggested.[69]

When genetic testing detects a melanoma predisposing mutation in a family, a screening cascade of individuals at risk is recommended. Mutation carriers are included in prevention and early-detection programs that include the use of sun protection, dermatologic screening, and self-skin examination, as those strategies result in earlier detection of thinner melanomas.[70-72] It has been demonstrated that melanoma genetic counseling has a positive impact on the improvement on total body skin examination and self-skin examination.[73] Furthermore, after melanoma genetic counseling unaffected members of high-risk melanoma families report improvements in daily routine sun protection, showing that genetic counseling may motivate sustained improvements in prevention behaviors.[74] Giving a genetic test result is perceived as more informative and

motivating for personal sun protection efforts than equivalent counseling based on family history alone.[75] Thus, it is very important for both melanoma patients and unaffected individuals to be included in genetic counseling programs and, when available, offer genetic testing. In families where no mutation is identified, it should be stressed that the family is still at increased risk of melanoma on the basis of the family history. These families should be managed according to family history.[19] More studies are needed to identify new genes involved in familial melanoma and to characterize better the already known genes in order to refine genetic counseling in melanoma.

MELANOMA PROGNOSTIC

Despite being the less frequent of the common skin cancers, melanoma is responsible for 75% deaths from skin cancer.[76] While, if diagnosed in early stages it can be surgically removed and cured, melanoma is the tumor with the highest metastatic capability for tumor volume, increasing 10% for each mm thickness.[77] Every year more than 60,000 deaths from melanoma occur worldwide,[4] being the crude mortality rate per 100,000 persons per year of 2.4 in males and 1.6 in females in Catalonia.[78]

Although the development of melanoma during childhood is rare, it can appear at any age and is the first most diagnosed cancer among patients from 25-29 years old, the second among 20-24-year-olds and the third solid tumor among 15-19-year-olds.[79] For this reason, melanoma is one of the cancers with more years of productive life lost and is the most expensive cancer when expressed in terms of cost per death in Europe.[80]

Clinicopathological prognostic factors

To date, histological features of the primary melanoma such as Breslow tumor thickness, mitotic rate, and ulceration, together with the Sentinel lymph node (SLN) biopsy, are important hallmarks of melanoma prognosis and staging.[77, 81-83]

Staging

Stage 0: includes melanomas not invasive to the dermis known as *in situ* melanoma. These lesions are thought to have none or extremely low metastatic potential and correlate with an excellent prognosis. However, a small risk of recurrence exists.[84]

Stage I and II: includes all invasive melanoma tumors without regional or distant metastasis evidence. Breslow thickness and ulceration have been clearly associated with

prognosis. According to the 8th edition staging system from the AJCC (American Joint Committee on Cancer),[81] for staging purposes Breslow thickness has been divided into four groups: T1 (≤ 1.0 mm), T2 (1.1-2.0 mm), T3 (2.1-4.0 mm) and T4 (> 4.0 mm). The presence of ulceration, defined as the absence of an intact epidermis overlying a major portion of the primary melanoma, provides de modification of "a" to "b" (worse prognosis with same tumor thickness). Tumor mitotic rate has also been recognized as an important prognostic factor in stage I-II patients.[85] In the previous AJCC staging edition,[77] the presence of 1 or more mitosis/mm^2 was also used to modify "a" to "b", but has been now modified.

Stage III: includes melanomas with the presence of regional metastasis in the lymph nodes, in-transit or tumor satellitosis. The number of regional metastases, the size (micro or macro) and location defines different staging subgroups.[81]

Stage IV: includes distant metastases in skin, subcutaneous or nodal, and visceral metastasis. Depending on the organ affected and the LDH (lactate dehydrogenase) blood levels different staging subgroups are defined. (A complete staging classification table is available in **Annex 5**).

Other relevant clinical characteristics not included in the current staging system

Age: Increasing age is also associated with a worse melanoma prognosis.[86] Balch and colleagues identified that with increasing age by decade, primary melanomas were thicker, exhibited higher mitotic rates, and were more likely to be ulcerated. However, age was independently associated with melanoma survival in stage III patients, suggesting that other factors than worse tumor prognostic features associated with older groups are implicated in this observed phenomenon.[87]

Gender: Males tend to have higher age, with thicker and more often ulcerated tumors.[6, 88] However, females have a better disease outcome both in early stages I/II and in advanced melanoma stages III/IV compared to males.[89, 90] This finding suggests that beyond behavioral differences, gender-specific biological factors are involved, at least in part, in disease outcome.[83]

Anatomic site: In our population, melanomas in the trunk are the most prevalent, with a higher prevalence in males (48.9%) than females (28.5%). Gender differences are also observed in the prevalence of melanomas in the extremities (males: 19.2%, females

41.7%) and head and neck – excluding face – (males 7.1%, females 3.3%).[6] Melanomas in the trunk or head and neck anatomic locations usually have a worse prognosis compared with melanomas in the extremities.[91-94]

Histological subtype: There are four predominant subtypes of melanoma: superficial spreading (SSM) with a prominent radial growth phase, nodular (NM) characterized by showing only vertical growth, acral lentiginous (ALM) found on palmar and plantar surfaces and mucosa, and lentigo maligna (LMM) common in chronic sun-exposed areas in elderly population (**Figure 7**). NM and ALM have the poorest survival rate.[94]

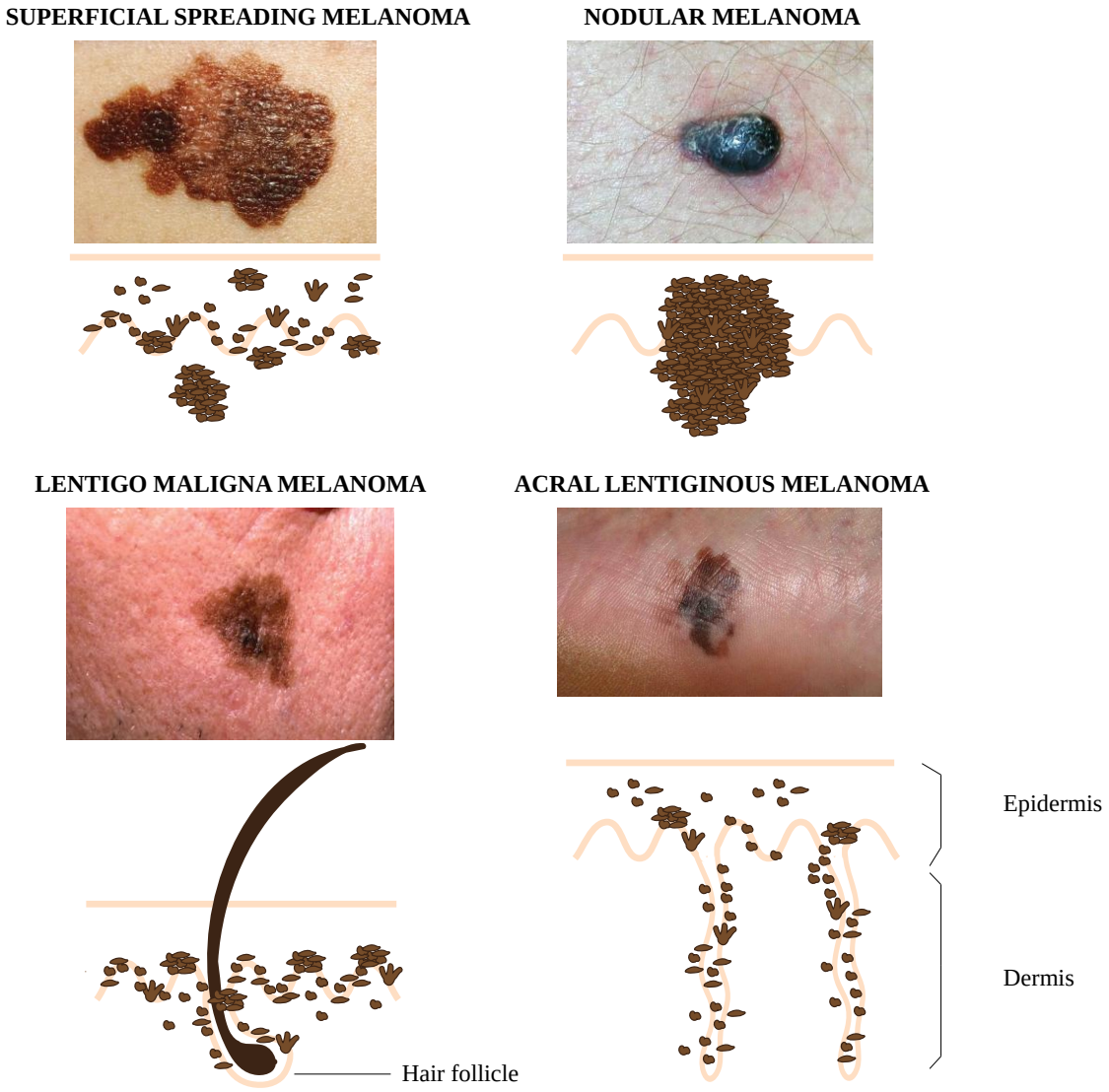

Figure 7. Main melanoma subtypes

Clinical image example for each of the four more common melanoma subtypes is shown. Below each clinical image, a schema of a skin section with the most representative traits for each subtype is drawn. Brown abnormal cells representative of different melanoma cell types have been used to represent the tumor cells. In **Annex 6** the equivalent figure with different nevi types is available.

Tumor-infiltrating Lymphocytes (TIL): The presence of TIL in the tumor correlates with a better melanoma prognosis, suggesting that the immune microenvironment affects tumor survival.[95]

Regression: Is the stromal reaction in front of the tumor, characterized by the presence of inflammation in early stages and fibrosis in late stages. The association of this feature with melanoma prognosis has been controversial. In some studies, regression has been associated with worse prognosis,[96] while in others shows a protective effect.[97] On one hand, it has been postulated that the presence of regression can result in an underestimation of the true Breslow thickness.[98] Moreover, although the significance is unclear, some authors have suggested that the presence of extensive regression may reflect an ineffective local immune response to the primary tumor[96] or a mechanism of selection of tumor subclones resistant to the immune system attack. Yet, the presence of early regression signs is an indicator of the attempt of the immune system to fight against the tumor.

Nevus count: The number of nevi decreases from middle age onward but this senescence can be delayed in patients with melanoma. Ribero and colleagues described that high nevus count was associated with favorable melanoma prognostic factors such as lower Breslow thickness, less ulceration, and lower mitotic rate. Melanoma-specific survival rate was higher in melanomas cases with a high nevus count compared to those with a low nevus count, even after adjusting for all known melanoma prognostic factors. This suggests a different biological behavior of melanoma tumors in patients with a high nevus count.[99]

Genetic prognostic factors

Clinicopathological information alone is not able to predict correctly the patient prognosis. Many efforts have been made to understand the role of tumor genetics and germline genetic variants into modulating melanoma survival. This thesis is focused on germline genetic factors study, thus only a brief view of the tumor genetic bases will be introduced.

BRAF (B-Raf Proto-Oncogene, Serine/Threonine Kinase) is an oncogene belonging to the MAPK pathway (RAS-RAF-MEK-ERK). *BRAF* activating mutations in amino acid Valine 600 are the most common somatic driver events identified in melanoma, found

in around 50% of melanomas.[100] For this reason, considering metastatic melanoma is highly resistant to conventional chemotherapy, BRAF/MEK targeted therapies have been developed, and represent nowadays one of the most common treatments for advanced melanomas. In some studies, activating mutations in *BRAF* have been associated with a poorer prognosis than wild-type *BRAF* melanomas.[101] However, treatment with BRAF targeted therapy may counteract the effects of the mutation. Other studies included both *BRAF* and *NRAS* (NRAS Proto-Oncogene, GTPase) mutations, which represents around 10-20% of somatic activating mutations in melanoma.[100, 102] In that case, the analysis found that *NRAS* but not *BRAF* mutations were associated with decreased overall survival in a multivariate analysis.[103] Nonetheless, for now, driver mutations analysis is only used for treatment selection.

Interestingly, there is an important relationship between genetic factors associated with the risk to develop melanoma and genetic factors modulating melanoma outcome. First of all, at the tumor level, high-risk melanoma susceptibility genes play also a role in tumor evolution. Large deletions of 9p, including 9p21 (the locus containing *CDKN2A*) have been associated with worse melanoma prognosis.[104, 105] In fact more than 50% invasive melanomas exhibit a total or partial loss of *CDKN2A*.[106, 107] Activating *TERT* promoter mutations induced by UVR in sun-exposed tumors and *TERT* amplification in ALM (non sun-exposed) also correlates with worse melanoma prognosis.[108, 109] At the germline level, the presence of *MC1R* variants, although increasing the risk to develop melanoma, are associated with a better disease outcome, as shown in two independent studies (Hazard Ratio [HR] = 0.64 and HR = 0.60, respectively).[110, 111] Loss of function variants in *MC1R* up-regulate oxidative stress-related pathways[112] and DNA damage,[113] favoring the apoptosis of damaged cells, which could explain in part this effect on prognosis. *PARP1* (Poly ADP-Ribose Polymerase 1) SNP (Single nucleotide polymorphism) rs2249844, which is in linkage with the variant associated with a reduced risk of melanoma development, is also implicated in melanoma survival (HR= 1.20).[114] Other variants in genes belonging to the nucleotide excision repair system have also been implicated with melanoma survival,[115] showing the relevance of the DNA damage and repair system for tumor survival. Several SNPs in *VDR* (Vitamin D Receptor[116] and the gene coding for vitamin D-binding protein[117], and in the interleukin locus, with the highest significance in *IL10* (Interleukin 10) (HR = 0.58),[118] have also been associated with melanoma survival. Finally, variants in genes from the Fanconi

anemia pathway, involved in DNA crosslink repair, and suggested to be associated with melanoma susceptibility,[67] are also implicated with melanoma prognosis.[119] Thus, we can observe a high interconnection between melanoma susceptibility and prognosis genes.

Other germline genetic variants playing a role in melanoma survival but not associated with melanoma susceptibility have been described. Those belong to other pathways: Hippo pathway, which control cell migration, development, and organ sizes in diverse species;[120] Notch signaling pathway, which is constitutively activated in melanoma, enhancing growth and aggressive metastatic potential of primary melanoma cells;[121] genes controlling angiogenesis;[122] epigenetic regulation,[123] and cell migration,[124] among other functions.[125]

Immune system in melanoma

We have already introduced the idea that the presence of lymphocytes infiltrating the tumor was a good prognosis sign. Moreover, some genetic variants related to the immune system (HLA and cytokines) have already been described as susceptibility and/or prognostic genetic biomarkers. These data allow us to think that immunity is extremely important for melanoma.

In fact, melanoma is a highly immunogenic tumor. For years, melanoma patients have presented spontaneous complete responses, even in metastatic cases,[126-128] sign of a spontaneous immunologic response against the tumor. The development of a good antitumor immune response lies in the coordination of the interactions between the host immunocompetent cells.[129] T cells express receptors that recognize specifically the antigens presented by the HLA (Human Leukocyte Antigen, also known as MHC, major histocompatibility complex antigens) molecules triggering the immune response that ends up eliminating the tumor cell. However, this system is highly regulated since it could be self-destructive if its activity could not be modulated. Thus, there are molecules able to activate or inhibit the immune response. T cells express inhibitory receptors such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4), TIM-3 (T-cell immunoglobulin domain and mucin domain 3) or PD-1(Programmed death-1), and other inhibitor molecules such as IDO (indoleamine-pyrrole-2,3-dioxygenase-1,2), LAG3 (lymphocyte-activation gene 3, CD223) o PD-L1 (Programmed Death-ligand 1). Melanoma can evade immune responses via several mechanisms including loss of

expression of HLA antigens, production of immunosuppressive cytokines, activation of regulatory T cells and expression of inhibitors for effector T cells (**Figure 8**).[130] The improvement in the comprehension of the tumor immunity has allowed the development of immunotherapies against the so-called immune checkpoints. Those therapies target CTLA-4, PD-1, and PD-L1 and there are clinical trials using inhibitors of TIM-3, LAG3, and IDO.[131, 132]

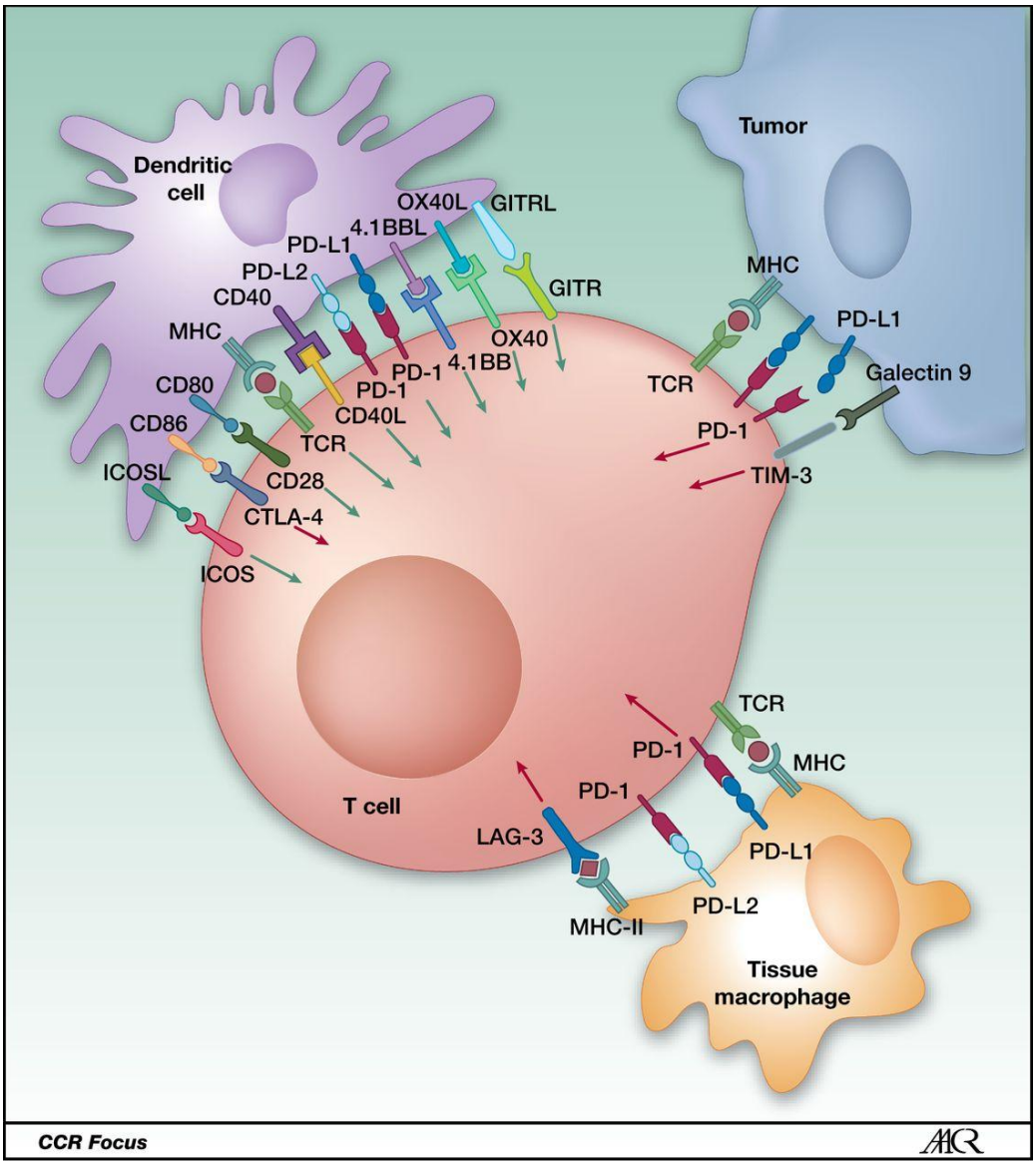

Figure 8. Tumor immune microenvironment

Co-stimulatory (green arrows) and co-inhibitory (red arrows) ligand-receptor interactions between a T cell and a dendritic cell, a tumor cell, and a macrophage, respectively, in the tumor microenvironment. Source: Ott et al. 2013.[133]

HYPOTHESIS AND OBJECTIVES

Under the hypothesis:

- The proper characterization of known melanoma risk genes in a specific population context will facilitate genetic counseling in melanoma.
- The use of genome-wide linkage can allow the identification of new melanoma susceptibility loci in our population.
- Melanoma susceptibility and nevus-related genes can play a role in melanoma prognosis.
- Variants in immune checkpoints genes can play a role in melanoma prognosis.

The main goals of this thesis dissertation are:

1. To characterize known risk genes in patients at high-risk to develop melanoma in Spain to refine genetic counseling.

   Specific aims to answer this goal include:

   a. To characterize families with *CDKN2A* mutations to refine genetic counseling.

   b. To study the role of *MITF* p.Glu318Lys in melanoma susceptibility in Spain.

   c. To study the role of telomere-related genes in melanoma susceptibility in Spain.

2. To identify new familial melanoma loci using genome-wide linkage analysis
3. To study the role of candidate genes in melanoma prognosis

   Specific aims to answer this goal include:

   a. To study the role in melanoma prognosis of melanoma susceptibility and nevi-related genes.

   b. To study the role in melanoma prognosis of immune checkpoints genes.

THESIS SUPERVISOR REPORT

Thesis title: CHARACTERIZATION OF GENETIC FACTORS ASSOCIATED WITH MELANOMA SUSCEPTIBILITY AND PROGNOSIS

Author: Míriam Potrony Mateu

Thesis supervisor: Susana Puig Sardà

Article 1

Title: Increased prevalence of lung, breast, and pancreatic cancers in addition to melanoma risk in families bearing the cyclin-dependent kinase inhibitor 2A mutation: implications for genetic counseling.

Authors: Potrony M, Puig-Butillé JA, Aguilera P, Badenas C, Carrera C, Malvehy J, Puig S.

Publication: J Am Acad Dermatol. 2014;71(5):888-95. doi: 10.1016/j.jaad.2014.06.036.

Impact Factor (JCR Science Edition 2014): 4.449 (1st decile, DERMATOLOGY)

Contribution of the doctoral student to the article: The doctoral student performed the statistical data analysis and was responsible for writing the original draft of the manuscript.

Article 2

Title: Prevalence of *MITF* p.E318K in patients with melanoma independent of the presence of *CDKN2A* causative mutations.

Authors: Potrony M, Puig-Butille JA, Aguilera P, Badenas C, Tell-Marti G, Carrera C, Javier Del Pozo L, Conejo-Mir J, Malvehy J, Puig S.

Publication: JAMA Dermatol. 2016;152(4):405-12. Doi: 10.1001/jamadermatol.2015.4356.

Impact Factor (JCR Science Edition 2016): 5.817 (1st decile, DERMATOLOGY)

Contribution of the doctoral student to the article: The doctoral student genotyped the samples of the study, performed the statistical data analysis and was responsible for writing the original draft of the manuscript.

Article 3

Title: *POT1* germline mutations but not *TERT* promoter mutations are implicated in melanoma susceptibility in a large cohort of Spanish melanoma families.

Authors: Potrony M, Puig-Butille JA, Ribera-Sola M, Iyer V, Robles-Espinoza CD, Aguilera P, Carrera C, Malvehy J, Badenas C, Landi MT, Adams DJ, Puig S

Publication: Manuscript under review in British Journal of Dermatology (first submission 11/05/2018)

Impact Factor (JCR Science Edition 2016): 4.706 (1st decile, DERMATOLOGY)

Contribution of the doctoral student to the article: The doctoral student participated in *POT1* germline variants selection, supervised Sanger sequencing confirmation and mRNA studies, supervised *TERT* promoter sequencing, performed statistical analysis, and was responsible for writing the original draft of the manuscript and figure preparation.

Article 4

Title: Genome-wide linkage analysis in Spanish melanoma-prone families identifies a new familial melanoma susceptibility locus at 11q.

Authors: Potrony M, Puig-Butille JA, Farnham JM, Giménez-Xavier P, Badenas C, Tell-Martí G, Aguilera P, Carrera C, Malvehy J, Teerlink CC, Puig S.

Publication: Eur J Hum Genet. 2018 (published online 30-April-2018). Doi: 10.1038/s41431-018-0149-8.

Impact Factor (JCR Science Edition 2016): 4.287 (1st quartile, GENETICS & HEREDITY)

Contribution of the doctoral student to the article: The doctoral student performed the genome-wide linkage data analysis and was responsible for writing the original draft of the manuscript.

Article 5

Title: *IRF4* rs12203592 functional variant and melanoma survival.

Authors: Potrony M, Rebollo-Morell A, Giménez-Xavier P, Zimmer L, Puig-Butille JA, Tell-Marti G, Sucker A, Badenas C, Carrera C, Malvehy J, Schadendorf D, Puig S.

Publication: Int J Cancer. 2017;140(8):1845-1849. Doi: 10.1002/ijc.30605.

Impact Factor (JCR Science Edition 2016): 6.513 (1st quartile, ONCOLOGY)

Contribution of the doctoral student to the article: The doctoral student participated in the clinical data cleaning, the genotyping process, performed the statistical data analysis and was responsible for writing the original draft of the manuscript.

Article 6

Title: Inherited functional variants of the lymphocyte receptor *CD5* influence melanoma survival.

Authors: Potrony M, Carreras E, Aranda F, Zimmer L, Puig-Butille JA, Tell-Martí G, Armiger N, Sucker A, Giménez-Xavier P, Martínez-Florensa M, Carrera C, Malvehy J, Schadendorf D, Puig S, Lozano F.

Publication: Int J Cancer. 2016;139(6):1297-302. Doi: 10.1002/ijc.30184.

Impact Factor (JCR Science Edition 2016): 6.513 (1st quartile, ONCOLOGY)

Contribution of the doctoral student to the article: The doctoral student participated in the clinical data cleaning, the genotyping process, performed the statistical data analysis and was responsible for writing the original draft of the manuscript.

None of the articles had been previously part of any other doctoral thesis.

Dr. Susana Puig Sardà

PUBLICATIONS

ARTICLE 1

Increased prevalence of lung, breast, and pancreatic cancers in addition to melanoma risk in families bearing the cyclin-dependent kinase inhibitor 2A mutation: implications for genetic counseling.

Potrony M, Puig-Butillé JA, Aguilera P, Badenas C, Carrera C, Malvehy J, Puig S.

J Am Acad Dermatol. 2014;71(5):888-95.

Aim

To investigate which clinical and familial history features are associated with the presence of germline *CDKN2A* mutations in Spanish patients with a high-risk of developing melanoma

Patients

702 melanoma patients visited at the Melanoma Unit – Hospital Clínic of Barcelona (January 1992 – June 2013):

- 236 sporadic MPM with ≥ 2 primary melanomas
- 466 familial cases, from 330 families with ≥ 2 cases

Methods

Sanger sequencing of *CDKN2A* exons 1α, 1β, 2 and 3, and *CDK4* exon 2.

Statistical association analyses.

Variables included in analyses

Presence of *CDKN2A* mutations

Age of onset

Number of primary melanomas

Number of cases in the family

Presence of any other cancer type in first- or second-degree relatives and specific cancers (pancreatic, colon, breast, lung and nephrourologic).

Main results

CDKN2A mutations were detected in 8.5% of sporadic MPM and 14.1% in melanoma-prone families. No mutations were detected in *CDK4*.

CDKN2A mutation was associated with:

- Increased with the number of cases in the family (10.9%, 23.4%, 36.4%, and 66.7% of families with 2, 3, 4, and 5 cases, respectively, $P = 0.001$)
- Increased number of MPM within the family (6.3%, 30.1%, and 40% of families with 0, 1, and 2 or more MPM patients, respectively, $P < 0.001$)
- Increased number of primary melanomas (12.6%, 21.2%, and 48.0% of MPM patients with 2, 3 or 4 or more primary melanomas, respectively, $P < 0.001$)
- Early age of onset (39.4 years old in *CDKN2A*-positive vs. 47.2% in *CDKN2A*-negative, $P < 0.001$)

Regarding the family history of cancer, *CDKN2A* mutations were associated with:

- Increased prevalence of any other cancer type (Prevalence ratio [PR] = 2.99, 95% CI 1.40-6.60, Adj. $P = 0.012$)
- Increased prevalence of pancreatic cancer (PR = 2.97, 95%CI 1.72-5.15, Adj. $P = 0.006$)
- Increased prevalence of lung cancer (PR = 3.04, 95%CI 1.93-4.80, Adj. $P < 0.001$)
- Increased prevalence of breast cancer (PR = 2.19, 95%CI 1.36-3.5, Adj. $P = 0.018$)

Increased prevalence of lung, breast, and pancreatic cancers in addition to melanoma risk in families bearing the cyclin-dependent kinase inhibitor 2A mutation: Implications for genetic counseling

Miriam Potrony,[a,c] Joan Anton Puig-Butillé, PhD,[a,b] Paula Aguilera, MD,[a,c] Celia Badenas, PhD,[a,b] Cristina Carrera, MD, PhD,[a,c] Josep Malvehy, MD, PhD,[a,c] and Susana Puig, MD, PhD[a,c]
Barcelona, Spain

Background: Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) is the major high-risk susceptibility gene for melanoma.

Objective: We sought to evaluate the effect of *CDKN2A* mutations in Spanish patients with a high risk of developing melanoma and the association with clinical and family history features.

Methods: A cross-sectional study design was used to analyze the *CDKN2A* impact in 702 Spanish patients with a high risk of developing melanoma.

Results: The *CDKN2A* mutation prevalence was 8.5% in patients with sporadic multiple primary melanoma and 14.1% in familial melanoma. Number of cases in the family, number of primary melanomas, and age of onset were associated with the presence of *CDKN2A* mutation. Having a *CDKN2A* mutation in the family increased the prevalence of other cancers (prevalence ratio [PR] 2.99, $P = .012$) and prevalence of pancreatic (PR 2.97, $P = .006$), lung (PR 3.04, $P < .001$), and breast (PR 2.19, $P = .018$) cancers but not nephrourologic or colon cancer.

Limitations: Smoking status was not assessed in the individuals with lung cancer.

Conclusions: Melanoma-prone families with mutations in *CDKN2A* have an increased prevalence of a broad spectrum of cancers including lung, pancreatic, and breast cancer. This information should be included in genetic counseling and cancer prevention programs for *CDKN2A* mutation carriers. (J Am Acad Dermatol 2014;71:888-95.)

Key words: breast cancer; *CDKN2A*; genetic counseling; lung cancer; melanoma; pancreatic cancer; prevention; risk; smoking.

Melanoma is a complex and heterogeneous disease, involving environmental, phenotypic, and genetic risk factors. Sunlight is the major environmental risk factor for melanoma[1] and phenotypic characteristics such as skin, eye, and hair color and the number of common and atypical cutaneous nevi are melanoma risk factors.[2,3]

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The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants 09/01393 and 12/00840; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) of the Instituto de Salud Carlos III, Spain; Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) grant 2009 SGR 1337 of the Catalan Government, Spain; European Commission under the Sixth Framework Program, contract no. LSHC-CT-2006-018702 (GenoMEL); and National Cancer Institute of the US National Institutes of Health (CA83115). Ms Potrony had a personal grant from the CIBERER of the Instituto de Salud Carlos III, Spain. The work was carried out at the Esther Koplowitz Center, Barcelona.
Ms Potrony and Dr Puig-Butillé contributed equally to this work.
Conflicts of interest: None declared.
Presented in part as an oral communication at the European Organization for Research and Treatment of Cancer melanoma group meeting in Majorca, Spain, on September 13-14, 2013.
Accepted for publication June 22, 2014.

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Published online July 24, 2014.
0190-9622/$36.00

http://dx.doi.org/10.1016/j.jaad.2014.06.036

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Approximately 5% to 10% of melanoma cases occur in a familial context.[4] To date, 2 high-penetrance genes have been implicated in melanoma susceptibility: cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase 4 (*CDK4*). Germline mutations in the *CDKN2A* gene have been observed in 20% to 40% of melanoma-prone families.[5] This gene codes for the tumor suppressor proteins p16 cyclin-dependent kinase 4 inhibitor A (p16INK4) and p14 alternative reading frame (p14ARF), both involved in cell cycle inhibition through different pathways.[6] Germline mutations in *CDK4*, an oncogene encoding one of the binding partners of p16INK4, are restricted to a few melanoma families.[7]

CAPSULE SUMMARY

- In addition to melanoma, other cancers have been observed in cyclin-dependent kinase inhibitor 2A—mutated melanoma-prone families.
- The study has identified an increased prevalence of pancreatic, breast, and lung cancer in melanoma-prone families carrying cyclin-dependent kinase inhibitor 2A mutations.
- These findings may improve the prevention strategies indicated for cyclin-dependent kinase inhibitor 2A mutation carriers.

In addition to melanoma, other cancers have been observed in *CDKN2A*-mutated melanoma-prone families and several studies have shown an increased risk of pancreatic cancer among these families.[8,9] In families carrying mutations, the relative risk of developing pancreatic cancer was 7.4 or 14.8 in studies from the United States[10] and Italy,[11] respectively. Furthermore, an increased risk of breast cancer has been observed in *CDKN2A*-mutated pedigrees.[12] In the first Spanish *CDKN2A*-mutated family, an increased risk for lung and breast cancers was also suggested.[13]

The identification of high-risk penetrance melanoma genes, which in turn are related to phenotypical characteristics of patients and number of cases within families, has allowed us to recommend genetic counseling for familial melanoma. Genetic counseling is a nondirected process offered to families to help them understand the meaning of the disease, the meaning of genetic susceptibility, the patterns of inheritance, the option of genetic testing, the understanding of all the possible results, and primary and secondary prevention.[14] To date, in low melanoma incidence populations the inclusion criteria for genetic testing in melanoma are: 2 (synchronous or metachronous) primary melanomas in an individual or families with at least 2 melanoma cases in first- or second-degree relatives.[15]

Also, the presence of pancreatic cancers among first- or second-degree relatives of the patients with melanoma has been considered as a selection criterion for genetic counseling.[15] Thus, identification of other malignancies related to *CDKN2A*-mutated families may be useful to refine the selection criteria and to improve preventive strategies. The aim of this study is to investigate which clinical and familial history features are associated with the presence of germline *CDKN2A* mutations in Spanish patients with a high risk of developing melanoma.

METHODS

Patients

A cross-sectional study design was used to analyze the *CDKN2A* impact in patients with a high risk of developing melanoma. Overall, 702 patients with melanoma were included in the study: 236 patients with sporadic multiple primary melanoma (SMP), and 466 patients with familial melanoma belonging to 330 high-risk melanoma-prone families with at least 2 melanoma cases (269 families with 2 melanoma cases, 47 families with 3 melanoma cases, 11 families with 4 melanoma cases, and 3 families with 5 melanoma cases). The patients included in the study were consecutively recruited from January 1992 to June 2013.

According to the number of tumors and the presence of other cases in the family, the set of patients included: patients with sporadic melanoma and multiple primaries (SMP, n = 236); melanoma patients with multiple primary melanoma (MPM) and familial history of melanoma (familial MPM [FMP], n = 115); and patients with melanoma and a single primary melanoma and family history of melanoma (n = 351).

The variables included in the analyses were age of onset, number of primary melanomas, number of melanoma cases within the family, and the presence of other cancers in first- and second-degree relatives of the patients with melanoma. We evaluated, specifically, whether first- and second-degree relatives developed pancreatic, colon, lung, nephrourologic (including kidney, bladder, or prostate), or breast cancers. We focused on those cancer types previously related to *CDKN2A* germline mutations such as pancreatic cancer and we have also included the most common cancers in Catalonia, Spain (colon, lung, breast, prostate, and bladder).[16] The cancer history was obtained from personal

Abbreviations used:

CDK4:	cyclin-dependent kinase 4
CDKN2A:	cyclin-dependent kinase inhibitor 2A
FMP:	familial multiple primary melanoma
MPM:	multiple primary melanoma
PR:	prevalence ratio
SMP:	sporadic multiple primary melanoma

interviews conducted the day of the melanoma diagnosis or during the follow-up.

Age of onset information was available for more than 90% of the patients and family history of other cancers was available in 90% of melanoma-prone families and 80% of SMP. A questionnaire about smoking habits was obtained from 172 individuals belonging to 75 melanoma-prone families. The set included 54 (31.4%) *CDKN2A* mutation carriers and 118 (68.6%) wild-type individuals. The smoking habits were classified as: never, former, or current smokers. The age of daily smoking onset and the number of cigarettes per day were also recorded for current and former smokers. Smoking habits from the general population from Catalonia were collected from the National Statistics Institute of Spain for the period July 2011 to June 2012.

All patients were selected from the melanoma unit database from the Hospital Clinic of Barcelona. The study was approved by the ethical committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

Mutational analysis

Genomic DNA was obtained from peripheral lymphocytes of the patients with melanoma included in the study according to the salting-out method.[17] The *CDKN2A* locus (exon 1α, 1β, 2, and 3) and *CDK4* exon 2 were amplified by polymerase chain reaction as previously described.[13,18,19]

Statistical analysis

The 2-sided χ^2 or Fisher exact test, as appropriate, was used to test for statistical significance in proportion comparison. Continuous variables, such as the age at diagnosis, were tested using the analysis of variance test. Analysis of familial history of other cancers was carried out by classifying the pedigrees as to absence or presence of a given type of cancer, and calculating the prevalence ratio (PR) and its 95% confidence intervals.

The functional effect of each genetic variant detected in *CDKN2A* was evaluated *in silico* using PolyPhen-2 software (http://genetics.bwh.harvard.edu/pph2/).[20] We excluded 1 SMP and 3 families because they carried mutations that did not alter the protein function. Furthermore, we excluded 1 other SMP and 1 family that carried mutations that cause an amino acid change in the protein because in silico analyses predicted them to be benign and they were not present in all the affected members in the family. The COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) was used to evaluate whether previously unreported germinal mutations were observed at the somatic level.

Bonferroni correction was applied in multiple analyses and an adjusted *P* value was obtained multiplying the test *P* value for the number of comparisons performed. The result was considered statistically significant if *P* value or adjusted *P* value, as appropriate, was less than .05. Software (SPSS 17.0, IBM Corp, Armonk, NY) was used for the statistical analyses.

RESULTS

CDKN2A and *CDK4* were tested in 702 patients with melanoma: 236 patients with SMP and 466 with familial melanoma belonging to 330 high-risk melanoma-prone families with at least 2 melanoma cases. Overall, 32 germline *CDKN2A* mutations were identified: 18 previously described mutations,[13,19,21-31] 9 mutations previously observed at the somatic level,[32,33] and 5 novel unreported mutations (data available on request). *CDK4* mutations were not observed.

Several sporadic cases or families were removed from the study based on different criteria. A SMP case carrying a predicted benign mutation that has not been previously described (p.G32R located in exon 1β); 1 family carrying a predicted benign mutation not previously described that did not segregate among cases (p.P11T located in exon 1α); 2 families with a synonymous change with unknown effect on the 3'UTR of protein p14ARF (c.369C>T located in exon 2), and 1 SMP patient and 1 family carrying intronic mutations.

CDKN2A mutations were detected in 8.5% (20 of 234) of patients with SMP and in 14.1% (46 of 326) of melanoma-prone families. In the set of families, the frequency of *CDKN2A* mutation differs according to the number of patients with melanoma within the family. *CDKN2A* mutation was found in 10.9% (29 of 265), 23.4% (11 of 47), 36.4% (4 of 11), and 66.7% (2 of 3) of families with 2, 3, 4, and 5 cases, respectively ($P = .001$). Also, statistically significant differences were observed according to the number of FMP in the family. Germline *CDKN2A* mutations were found in 6.3% (14 of 224) of families without FMP cases and in 30.1% (25 of 83) and 40% (6 of 15) of families with 1 or 2 FMP cases, respectively ($P < .001$).

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Table I. Number of primary melanomas in patients with multiple primary melanoma according to cyclin-dependent kinase inhibitor 2A status

	CDKN2A+		*CDKN2A* WT		
Analysis group	**No.**	**%**	**No.**	**%**	***P* value**
All MPM					
2	34	12.6	236	87.4	**<.001**
3	11	21.2	41	78.8	
≥4	12	48.0	13	52.0	
SMP					
2	12	6.3	179	93.7	**.015**
3	5	15.6	27	84.4	
≥4	3	27.3	8	72.7	
FMP					
2	22	27.8	57	72.2	**.033**
3	6	30.0	14	70.0	
≥4	9	64.3	5	35.7	

Statistically significant *P* values are set in bold.
CDKN2A, Cyclin-dependent kinase inhibitor 2A; *FMP*, familial multiple primary melanoma; *MPM*, multiple primary melanoma; *SMP*, sporadic multiple primary melanoma; *WT*, wild type; +, mutation.

Table II. Age at diagnosis of first melanoma according to cyclin-dependent kinase inhibitor 2A status

Analysis group (patients)	**No.**	**Mean age at diagnosis, y**	**SD**	**Adjusted *P***
All				
CDKN2A+	90	39.4	12.8	**<.001**
CDKN2A WT	546	47.2	16.5	
Total	636	46.1	16.3	
Missing data	58			
Familial melanoma				
CDKN2A+	72	40.1	13.6	**.040**
CDKN2A WT	350	45.3	15.9	
Total	422	44.4	15.6	
Missing data	38			
All MPM				
CDKN2A+	53	38.1	11.9	**<.001**
CDKN2A WT	266	50.0	17.1	
Total	319	48.0	16.9	
Missing data	28			
SMP				
CDKN2A+	18	35.8	8.5	**<.001**
CDKN2A WT	196	50.6	17.2	
Total	214	49.4	17.1	
Missing data	20			

Statistically significant *P* values are set in bold.
Bonferroni correction was used.
CDKN2A, Cyclin-dependent kinase inhibitor 2A; *MPM*, multiple primary melanoma; *SMP*, sporadic multiple primary melanoma; *WT*, wild type; +, mutation.

In the subgroup of patients with MPM, we evaluated whether the presence of a *CDKN2A* mutation was related to the number of melanoma developed independent of the familial history of melanoma. We found that the number of tumors correlates with the presence of *CDKN2A* mutations, observing 12.6% of positive mutation carriers in cases with 2 melanomas, and up to 48.0% of mutation carriers in patients who develop at least 4 melanomas. These differences were also detected in both SMP and FMP cases (Table I).

We observed that the presence of *CDKN2A* mutations also modulates the age of onset among the set of patients. Overall, patients with melanoma and *CDKN2A* mutations showed lower age of onset compared with wild type cases (adjusted $P < .001$) (Table II). Such differences were also found when the analyses were focused on patients with familial melanoma (adjusted $P = .040$), all MPM (adjusted $P < .001$), and SMP (adjusted $P < .001$).

Finally, the overrepresentation of other malignances in *CDKN2A*-mutated families was assessed combining the information from the 326 melanoma-prone families and from those families with a SMP case. Overall, the presence of relatives that develop other cancer types was more frequently reported by patients with melanoma carrying *CDKN2A* mutations. We found that the PR of other cancers in germline *CDKN2A* mutated pedigrees was 2.98 (adjusted $P = .012$). The analyses according to cancer type showed an increased presence of pancreatic cancer (PR 2.97, adjusted $P = .006$), lung cancer (PR 3.04, adjusted $P < .001$), and breast cancer (PR 2.19, adjusted $P = .018$) in first- and second-degree relatives of patients with melanoma carrying *CDKN2A* mutations, compared with the wild type. In contrast, no differences were observed in the presence of nephrourologic or colon cancer (Table III). In the analyses restricted to melanoma-prone families (Table III), the association between the presence of pancreatic cancer and lung cancer and *CDKN2A* mutation in the family remained statistically significant (PR 3.26, adjusted $P = .012$ and PR 3.17, adjusted $P < .001$; respectively).

To evaluate whether the increased number of patients with lung cancer within *CDKN2A*-mutant families could be associated with differences related to smoke exposure, we analyzed the smoking habits in 172 individuals from 75 melanoma-prone families. Overall, we did not observe differences in smoking habits, number of cigarettes, age of daily smoking onset, or the number of cigarettes/day between *CDKN2A* mutation carriers vs non-*CDKN2A* mutation carriers or between patients with melanoma and nonaffected individuals. Furthermore, no differences were observed in the smoking habits between the individuals included in our database compared with

Table III. Families with presence/absence of other cancers in first- and second-degree relatives of the patient with melanoma according to the family cyclin-dependent kinase inhibitor 2A status

Analysis including SMP families and melanoma-prone families with at least 2 melanoma cases							
	CDKN2A+ (n = 66)		*CDKN2A* WT (n = 494)				
Other solid tumors in the family	**No.**	**%**	**No.**	**%**	**PR**	**95% CI**	**Adjusted *P***
Other cancers (all)							
Presence	54	88.5	292	69.7	**2.99**	1.40-6.40	**.012**
Absence	7	11.5	127	30.3			
Missing data	5		75				
Pancreatic cancer							
Presence	11	18.0	22	5.3	**2.97**	1.72-5.15	**.006**
Absence	50	82.0	396	94.7			
Missing data	5		76				
Nephrourologic cancer*							
Presence	10	16.4	41	9.8	1.64	0.89-3.03	.738
Absence	51	83.6	376	90.2			
Missing data	5		77				
Lung cancer							
Presence	24	39.3	60	14.4	**3.04**	1.93-4.80	**<.001**
Absence	37	60.7	357	85.6			
Missing data	5		77				
Breast cancer							
Presence	20	32.8	67	16.1	**2.19**	1.36-3.55	**.018**
Absence	41	67.2	349	83.9			
Missing data	5		77				
Colon cancer							
Presence	9	14.3	66	15.8	0.90	0.47-1.74	1.000
Absence	54	85.7	351	84.2			
Missing data	3		77				
Analysis focused only on melanoma-prone families with at least 2 melanoma cases							
	CDKN2A+ (n = 46)		*CDKN2A* WT (n = 280)				
Other solid tumors in the family	**No.**	**%**	**No.**	**%**	**PR**	**95% CI**	**Adjusted *P***
Other cancers (all)							
Presence	37	86.0	177	70.5	2.31	1.01-6.37	.246
Absence	6	14.0	74	29.5			
Missing data	3		29				
Pancreatic cancer							
Presence	9	20.9	13	5.2	**3.26**	1.92-12.14	**.012**
Absence	34	79.1	237	94.8			
Missing data	3		30				
Nephrourologic cancer*							
Presence	9	20.9	26	10.4	1.94	1.02-3.70	.426
Absence	34	79.1	223	89.6			
Missing data	3		31				
Lung cancer							
Presence	18	41.9	36	14.5	**3.17**	1.87-5.39	**<.001**
Absence	25	58.1	213	85.5			
Missing data	3		31				
Breast cancer							
Presence	14	32.6	41	16.5	2.08	1.18-3.67	.114
Absence	29	67.4	208	83.5			
Missing data	3		31				
Colon cancer							
Presence	8	17.8	38	15.4	1.16	0.58-2.32	1.000
Absence	37	82.2	209	84.6			
Missing data	1		33				

Statistically significant *P* values are set in bold.
Bonferroni correction was used.
CDKN2A, Cyclin-dependent kinase inhibitor 2A; *CI*, confidence interval; *PR*, prevalence ratio; *SMP*, sporadic multiple primary melanoma; *WT*, wild type; +, mutation.
*Kidney, bladder, or prostate cancer.

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those observed in the general population (data available on request).

DISCUSSION

In this study we have explored the effect of germline mutations in *CDKN2A*, which is the major high-risk melanoma susceptibility gene, in the largest Spanish cohort to our knowledge of patients at high risk (SMP and familial melanoma cases). Overall, we found a slightly increased prevalence of *CDKN2A* mutations in melanoma-prone families than in SMP, consistent with that reported in similar studies from other Mediterranean areas.[34-36]

Previous studies have found an association between the presence of *CDKN2A* mutations within a family and clinical features of the family such as an increased number of cases, or in patients with melanoma, such as an increased number of tumors or a decreased age of onset.[5,37] We also observed an increased number of melanoma cases or number of FMP in Spanish *CDKN2A*-mutated families, an increased number of tumors, and a younger age of onset in patients carrying *CDKN2A* mutations.

The presence of other types of cancer in melanoma-prone families was evaluated regarding the germline status of *CDKN2A*. Overall, the families in which the melanoma cases carried *CDKN2A* mutations showed an increased presence of individuals with other types of cancer. Analyses focused on specific cancer types revealed an association between the presence of germline mutations and cases of pancreatic, lung, and breast cancer. An increased risk of pancreatic cancer was observed in *CDKN2A* mutated families of Caucasian origin.[5,8,10-12,28,34] A multicenter study conducted in a large set of families with 3 or more melanoma cases found a highly increased risk of pancreatic cancer in European families (odds ratio 8.21, 95% confidence interval 2.39-28.24).[5] We detected an increased risk for pancreatic cancer in Spanish melanoma families, even including families with 2 melanoma cases as also reported in French families.[34] Although previous data suggest that germline *CDKN2A* mutations may result in an increased risk of developing other types of cancer,[13] the role of *CDKN2A* in the risk for other cancer types has been less explored. To our knowledge, our study is the largest single-center data set in which the prevalence of other cancers beyond melanoma in *CDKN2A*-mutant families was evaluated. We observed a strong association between the presence of *CDKN2A* mutations and cases of lung cancer within the family. There is a previous study suggesting the association between respiratory cancer and p16-Leiden *CDKN2A* mutation[38] and our group reported a melanoma-prone family in which *CDKN2A* mutations were also present in lung cancer–affected individuals.[13]

In contrast to the association observed with pancreatic or lung cancer, the statistically significant association with breast cancer was restricted to the analyses combining the information from melanoma-prone families and from those families with SMP. The risk of breast cancer in melanoma-prone families carrying *CDKN2A* mutations has been reported previously in North-European populations.[12]

Genetic counseling is increasingly being offered to patients with cancer and/or to their healthy relatives. The genetic testing offered to patients at high risk to develop melanoma may allow us to detect *CDKN2A* mutation carriers who would then be encouraged to practice strategies for melanoma prevention, such as ultraviolet protection, and strategies for early detection. In this study we have found that *CDKN2A*-mutated melanoma families have an increased prevalence of pancreatic, lung, and breast cancer. The effects of cigarette smoking on lung cancer risk is well documented, but also pancreatic cancer[39] and breast cancer[40] have been associated with this risk factor. Furthermore, it has been reported that pancreatic cancer penetrance is higher in smoking *CDKN2A* mutation carriers than in nonsmoker carriers.[41] Our results indicated that the increased prevalence of these cancers observed in *CDKN2A*-mutated families could be explained by genetic factors, when they are exposed to the same environmental factors as the general population.

In conclusion, we have evaluated clinical and family history features related to the presence of germline *CDKN2A* mutations in patients at high risk to develop melanoma and we have observed an increased prevalence of lung, pancreatic, and breast cancer in families carrying *CDKN2A* germline mutations. The data reported in this study may be useful to refine genetic counseling in melanoma and encourage improving cancer prevention programs for *CDKN2A* mutation carriers by adding the recommendation of avoiding smoking in the programs, which already include sun-exposure protection advice and routine total body examination for melanoma early detection. Further studies are needed to identify the best early detection strategies for other cancers in *CDKN2A*-mutant families.

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Data available upon request

Table S1. *CDKN2A* mutations detected in Spanish high-risk melanoma patients

CKDN2A region	Nucleotide change	p16INK4 aminoacid change	p14ARF aminoacid change	p16INK4 evaluation*	p14ARF evaluation*	No. Spanish SMP	No. Spanish melanoma-prone families	Previously described in melanoma patients	Reference Δ
1 beta	c.60ins16	-	p.R21RfsX46	-	frameshift	1	0	YES	21
1 beta	c.94G>A	-	p.G32R	-	PS: 0.006	1	0	NO	-
5'UTR 1 alpha	c.-25C>T	-	-	Functional defects	-	1	0	YES	22
5'UTR 1 alpha	c.-34G>T	-	-	Functional defects	-	3	2	YES	23
1 alpha	c.13G>A	p.A5T	-	PS: 0.049†	-	0	1	NO§	COSMIC
1 alpha	c.31C>A	p.P11T	-	PS: 0.000¥	-	0	1	YES	-
1 alpha	c.104G>A	p.G35E	-	PS: 1.000	-	2	0	YES	24
1 alpha	c.106delG	p.A36RfsX17	-	frameshift	-	0	5	YES	25
1 alpha	c.116A>G	p.N39S	-	PS: 0.864	-	0	1	NO	-
1 alpha	c.131dup	p. Y44X	-	frameshift	-	1	0	NO§	COSMIC
1 alpha	c.149A>G	p.Q50R	-	PS: 0.997	-	0	1	YES	24
Intron 1	IVS1+37 C>G	-	-	unknown	unknown	1	0	YES	26
2	c.164G>T	p.G55V	-	PS: 1.000	NO	0	1	NO	-
2	c.176T>G	p.V59G	p.S73R	PS: 0.996	PS: 0.000	0	3	YES	19
2	c.194T>C	p.L65P	-	PS: 1.000	-	0	1	YES	28
2	c.212A>G	p.N71S	-	PS: 0.989	NO	0	2	YES	27

CKDN2A region	Nucleotide change	p16INK4 aminoacid change	p14ARF aminoacid change	p16INK4 evaluation*	p14ARF evaluation*	No. Spanish SMP	No. Spanish melanoma-prone families	Previously described in melanoma patients	Reference Δ
2	c.238C>T	p.R80X	p.P94L	frameshift	PS: 0.985	0	1	NO§	32
2	c.241C>T	p.P81S	p.T95I	PS: 1.000	PS: 0.933	0	1	NO§	COSMIC
2	c.250G>T	p.D84Y	p.R98L	PS: 1.000	PS: 1.000	0	1	YES	23
2	c.259C>T	p.R87W	p.P101L	PS: 0.675	PS: 0.987	1	1	YES	23
2	c.262G>T	p.E88X	p.G102V	frameshift	PS: 0.993	1	1	YES	29
2	c.295C>T	p.R99W	p.P113L	PS: 1.000	PS: 0.913	0	1	NO§	COSMIC
2	c.301G>A	p.G101R	p.R115Q	PS: 1.000	PS: 0.998	1	1	YES	30
2	c.301G>T	p.G101W	p.R115L	PS: 1.000	PS: 0.973	7	17	YES	27
2	c.305C>T	p.A102V	-	PS: 1.000	-	0	1	NO§	COSMIC
2	c.318G>A	-	p.A121T	-	PS: 0.979	1	1	NO§	COSMIC
2	c.335G>C	p.R112P	-	PS: 1.000	-	0	1	NO§	32
2	c.358delG	p.E120SfsX21	-	frameshift	-	0	1	YES	13
2	c.359_365del	p.E120AfsX24	-	frameshift	-	0	1	NO	-
2	c.369C>T	p.H123H	-	-	unknown	0	2	NO	-
2	c.379G>A	p.A127S	-	PS: 0.982	-	1	0	YES	31
Intron 2	IVS2-2A>G	-	-	unknown	unknown	0	1	NO§	33

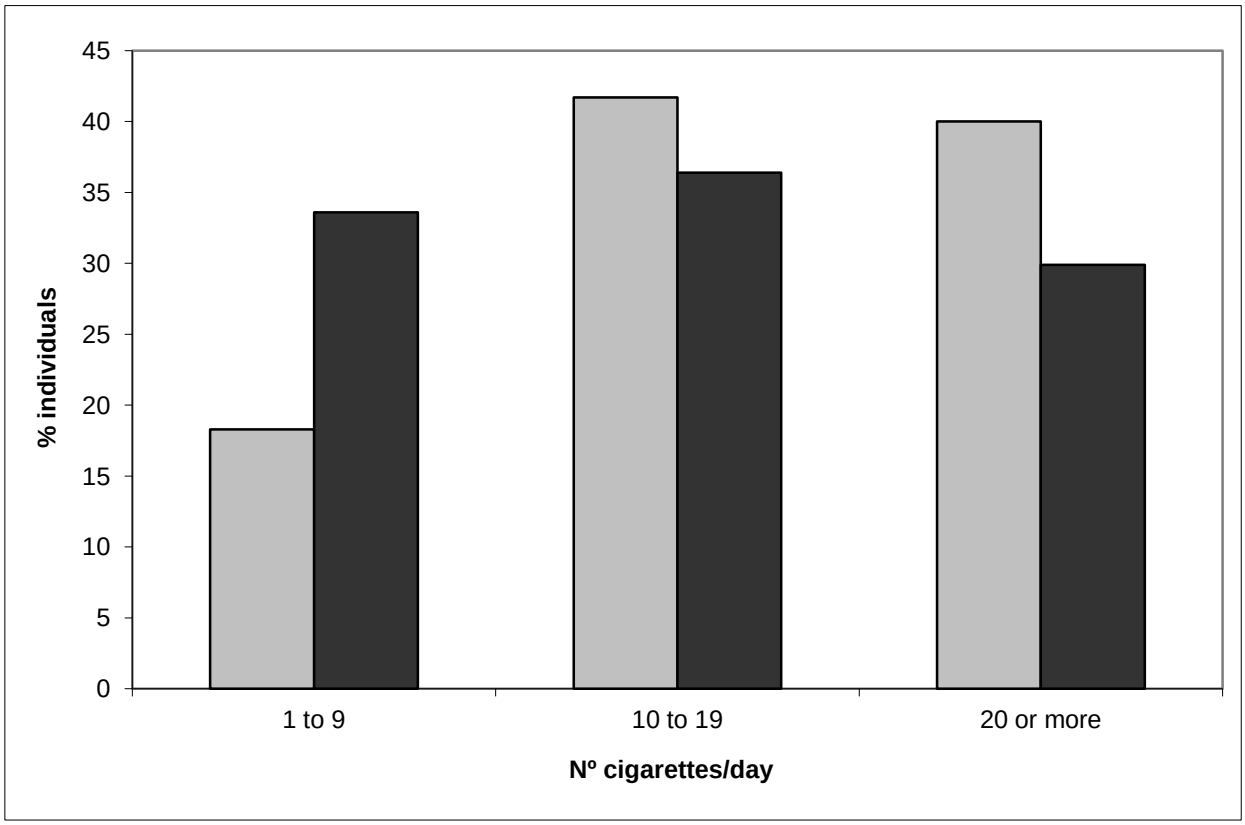
[*:] The effect of the variants are indicated as: "functional defects" when the variants promote functional protein alterations observed in prior experimental studies (Bisio A, Nasti S, Jordan JJ, et al: Functional analysis of CDKN2A/p16INK4a 5'-UTR variants predisposing to melanoma. Hum Mol Genet 19:1479-91, 2010); "frameshift" when the variant is a frameshift mutation; "PS" indicates the Polyphen-2 score (the score ranging from 0.000 to 1.000, 0.000 being the most benign prediction and 1.000 the most damaging prediction) and "unknown" when the variant has not been evaluated *in-silico*. [†] The mutation segregates in the family. [¥]The mutation was only found in one of the two melanoma patients from the family. [§]Previously undescribed as germline mutations in high-risk melanoma patients but reported as somatic mutation in cancer. [Δ]Number indicates the reference of the article reporting the mutation; COSMIC indicates that the mutation is reported in the COSMIC database; - indicates that the mutation has not been previously described. SMP: Sporadic multiple primary melanoma patient

Table S2. Smoking habits of Barcelona melanoma-prone families vs. Catalonian general population

Smoking habits	Barcelona Melanoma-prone families		Catalonian General population	
	N	%	N	%
Never Smoked	103	59.9	3315	54.6
Former Smoker	36	20.9	1221	20.1
Current Smoker	33	19.2	1534	25.3
Total	172	-	6070	-

The table shows the comparison of smoking habits between melanoma-prone families and general population of our geographic area. Data for the general population habits was collected from the Spanish National Statistics Institute (www.ine.es). The p-value obtained for the test comparison was 0.185.

Figure S1. Distribution of the number of cigarettes/day



The figure shows the distribution of the number of cigarettes/day of our individuals (light grey) and the general population of our geographic region (dark grey). The p-values of the proportion comparison are the following: 1 to 9 ($p<0.05$), 10 to 19 ($p=0.4933$) and 20 or more ($p=0.1283$).

ARTICLE 2

Prevalence of *MITF* p.E318K in patients with melanoma independent of the presence of *CDKN2A* causative mutations.

Potrony M, Puig-Butille JA, Aguilera P, Badenas C, Tell-Marti G, Carrera C, Javier Del Pozo L, Conejo-Mir J, Malvehy J, Puig S.

JAMA Dermatol. 2016;152(4):405-12.

Aim

To evaluate the role of the *MITF* p.Glu318Lys variant in Spanish patients with melanoma and assess the association of this variant with clinical and phenotypic features.

Patients

531 melanoma patients visited at the Melanoma Unit – Hospital Clínic of Barcelona (January 1992 – June 2014):

- 271 MPM p16INK4A-wild-type with ≥ 2 primary melanomas (212 sporadic and 59 with family history of melanoma)
- 69 probands of melanoma-prone families (with ≥ 2 cases) with p16INK4A mutation
- 499 cancer-free controls age and sex matched with the melanoma cases

Methods

The *MITF* variant p.Glu318Lys (rs149617956) was analyzed using Custom TaqMan SNP-Genotyping Assays.

Statistical association analyses.

Variables included in analyses

Presence of *MITF* p.Glu318Lys

Age of onset

Number of primary melanomas

Primary melanoma features (Breslow thickness, histological subtype, anatomic site)

Phenotypic characteristics (eye and hair color, skin phototype, nevus count)

Family history of pancreatic cancer

Personal history of other cancers in *MITF* p.Glu318Lys carriers

Main results

MITF p.Glu318Lys was detected in 1.9% of p16INK4A-wild-type melanoma patients. When focusing only in MPM the variant prevalence increased to 2.6%, similar to the prevalence in p16INK4A mutation carriers 2.9%.

MITF p.Glu318Lys variant was associated with:

- Increased risk to develop melanoma (OR [Odds Ratio] = 3.3, 95% CI 1.43-7.43, $P < 0.01$) and specially MPM (OR = 4.5, 95% CI 1.83-11.01, $P < 0.01$).
- High nevi count (> 200 nevus) (OR = 8.4, 95% CI = 2.14-33.19, $P < 0.01$), specially in MPM (OR = 12.4, 95% CI 2.58-59.70, $P < 0.01$).
- Two fast growing melanomas were detected among patients during follow-up.

Research

Original Investigation

Prevalence of *MITF* p.E318K in Patients With Melanoma Independent of the Presence of *CDKN2A* Causative Mutations

Miriam Potrony, MSc; Joan Anton Puig-Butille, PhD; Paula Aguilera, MD; Celia Badenas, PhD; Gemma Tell-Marti, MSc; Cristina Carrera, MD, PhD; Luis Javier del Pozo, MD; Julian Conejo-Mir, MD; Josep Malvehy, MD, PhD; Susana Puig, MD, PhD

IMPORTANCE The main high-penetrance melanoma susceptibility gene is *CDKN2A*, encoding p16INK4A and p14ARF. The gene *MITF* variant p.E318K also predisposes to melanoma and renal cell carcinoma. To date, the prevalence of *MITF* p.E318K and its clinical and phenotypical implications has not been previously assessed in a single cohort of Spanish patients with melanoma or in p16INK4A mutation carriers.

OBJECTIVES To evaluate the prevalence of *MITF* p.E318K in Spanish patients with melanoma and assess the association with clinical and phenotypic features.

DESIGN, SETTING, AND PARTICIPANTS A hospital-based, case-control study was conducted at the Melanoma Unit of Hospital Clinic of Barcelona, with *MITF* p.E318K genotyped in all patients using TaqMan probes. We included 531 patients: 271 patients with multiple primary melanoma (MPM) without mutations affecting p16INK4A (wild-type p16INK4A); 191 probands from melanoma-prone families with a single melanoma diagnosis and without mutations affecting p16INK4A, and 69 probands from different families carrying *CDKN2A* mutations affecting p16INK4A. A population-based series of 499 age- and sex-matched cancer-free individuals from the Spanish National Bank of DNA were included as controls. Patients were recruited between January 1, 1992, and June 30, 2014; data analysis was conducted from September 1 to November 30, 2014.

MAIN OUTCOMES AND MEASURES The genetic results of the *MITF* p.E318K variant were correlated with clinical and phenotypic features.

RESULTS Among the 531 patients, the prevalence of the *MITF* p.E318K variant was calculated among the different subsets of patients included and was 1.9% (9 of 462) in all melanoma patients with wild-type p16INK4A, 2.6% (7 of 271) in those with MPM, and 2.9% (2 of 69) in the probands of families with p16INK4A mutations. With results reported as odds ratio (95% CI), the *MITF* p.E318K was associated with an increased melanoma risk (3.3 [1.43-7.43]; $P < .01$), especially in MPM (4.5 [1.83-11.01]; $P < .01$) and high nevi count (>200 nevi) (8.4 [2.14-33.19]; $P < .01$). Two fast-growing melanomas were detected among 2 *MITF* p.E318K carriers during dermatologic digital follow-up.

CONCLUSIONS AND RELEVANCE In addition to melanoma risk, *MITF* p.E318K is associated with a high nevi count and could play a role in fast-growing melanomas. Testing for *MITF* p.E318K should not exclude patients with known mutations in p16INK4A. Strict dermatologic surveillance, periodic self-examination, and renal cell carcinoma surveillance should be encouraged in this context.

Editorial page 375

Supplemental content at jamadermatology.com

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JAMA Dermatol. 2016;152(4):405-412. doi:10.1001/jamadermatol.2015.4356
Published online December 9, 2015.

Approximately 8% to 12% of melanoma cases occur in a familial context.[1] The main high-penetrance gene implicated in melanoma susceptibility is *CDKN2A* (OMIM code: 600160; GenBank accession number: NM_000077.4 [p16INK4A] and NM_058195.3 [p14ARF]). The gene encodes 2 tumor suppressor proteins: p16INK4A, which promotes cell-cycle arrest and plays a role in senescence, and p14ARF, which acts through p53-regulating apoptosis.[2,3] Germline *CDKN2A* mutations are found in 20% to 40% of melanoma-prone families[4] and in 8% to 16% of patients with multiple primary melanomas without other cases in the family.[5] Patients with melanoma carrying *CDKN2A* mutations have a younger age of onset and a higher number of primary melanomas, and the presence of *CDKN2A* mutations is associated with dysplastic nevi.[4-7]

The *MC1R* gene (OMIM: 155555; GenBank: NM_002386) controls the pigmentation process and is a moderate-risk gene for melanoma susceptibility.[8,9] Loss of function variants in *MC1R* impairs the ability to activate the pigmentation pathway resulting in the red-hair color (RHC) phenotype. The RHC phenotype is characterized by fair pigmentation (fair skin, red hair, and freckles) and by sun sensitivity (poor tanning response and solar lentigines).[10] These variants increase the risk of melanoma with an odds ratio (OR) between 1.5 and 4.1.[8,10,11] The association between *MC1R* variants and melanoma is stronger in individuals with dark skin or few nevi.[12,13] Therefore, there might be a modest benefit to measure *MC1R* genotype for melanoma risk prediction, in addition to clinically measured pigmentation characteristics and nevi count.[12] A rare functional variant in *MITF*, p.E318K (rs149617956), was identified in 2 independent studies.[14,15] This variant may be considered as a moderate-risk allele in melanoma.[14-17] The master regulator gene of melanocyte development and differentiation is *MITF*, and it is also associated with melanoma development and progression.[18] The presence of *MITF* p.E318K predisposes to both familial and sporadic melanoma susceptibility, and/or renal cell carcinoma (RCC), and/or pancreatic cancer.[14-16] *MITF* p.E318K occurs at a conserved small ubiquitinlike modifier position, and this variant decreases the amount of small ubiquitinlike modifier-modified MITF forms.[14] The small ubiquitinlike modifier of MITF represses its transcriptional activity; therefore, p.E318K increases MITF transcriptional activity and may result in the upregulation of distinct sets of genes. Furthermore, this variant promotes invasive and tumorigenic behaviors in melanoma and RCC cells and might favor a phenotypic switch of melanoma cells toward a tumor-initiating cell phenotype.[14]

To our knowledge, the role of the *MITF* p.E318K has not been previously explored in Spanish patients with melanoma. The aim of this study was to evaluate the role of the *MITF* p.E318K variant in Spanish patients with melanoma and assess the association of this variant with clinical and phenotypic features.

Methods

Patients

A total of 531 patients at high risk of melanoma, recruited from January 1, 1992, to June 30, 2014, at the Melanoma Unit of Hospital Clinic of Barcelona, were included in the study. Patients were grouped into 3 different subsets. The first set was composed of 271 individuals (51%) with multiple primary melanoma (MPM) (212 sporadic MPM and 59 familial MPM) who did not carry mutations in *CDKN2A* affecting the p16INK4A protein (hereinafter referred to as wild-type p16INK4A). One patient from this set carried a *CDKN2A* mutation affecting p14ARF, and 75 patients were previously included in the Bertolotto et al[14] study. The second set consisted of 191 probands (36%) from melanoma-prone families, with at least 2 melanoma cases, with a single melanoma diagnosis (all wild-type p16INK4A). The third set contained 69 probands (13%) from families bearing *CDKN2A* mutations affecting p16INK4A, independent of the number of primary melanomas.

Clinical and phenotypic characteristics were collected for most of the 531 patients, including the number of primary melanomas (99%), age of onset (90%), melanoma subtype (76%), melanoma location (84%), Breslow thickness (74%), eye and hair color (75%), skin phototype (80%), and nevus count (72%). The familial history of pancreatic cancer was obtained from 80% of the patients, and personal history of other cancers was supplied by the patients carrying the variant.

The study was approved by the ethics committee of the Hospital Clinic of Barcelona, and patients provided written informed consent. Participants did not receive financial compensation.

A population-based series of 499 cancer-free individuals recruited at the Spanish National Bank of DNA were used as controls. The controls were sex and age matched with a group of consecutively recruited individuals with sporadic melanoma in the Melanoma Unit of Hospital Clinic of Barcelona from January 1998 to December 2013. The mean (SD) age in the control group was 52.2 (18.0) years. Overall, 269 of the 499 patients were women (53.9%) and 230 were men (46.1%).

MITF p.E318K Genotyping

The *MITF* variant p.E318K (rs149617956) was analyzed (Custom TaqMan SNP Genotyping Assays) according to manufacturer's recommendations in all of the patients and the control group. The process was carried out using polymerase chain reaction (7900HT Fast Real Time PCR System; Applied Biosystems) and SDS, version 2.4, software (Applied Biosystems).

Statistical Analysis

The prevalence of *MITF* p.E318K was assessed in all melanoma patients with wild-type p16INK4A and the *CDKN2A* mutation carrier set. In a French cancer-free and Italian control population, the frequency of carriers was 0.6% (14 of 2205).[14,16] The risk conferred by *MITF* p.E318K to melanoma development in the Spanish patients with wild-type p16INK4A melanoma was evaluated by comparing our group of patients with the Spanish and previously reported French and Italian controls together since there were no statistically significant differences between them. Clinical and phenotypic characteristics were analyzed regarding the presence of the p.E318K variant in patients with wild-type p16INK4A. Odds ratios and

Table 1. Melanoma Risk and Phenotypic Features According to the Presence of *MITF* p.E318K in Patients With Wild-Type p16INK4A

Characteristic	No. (%)		OR (95% CI)	*P* Value
	MITF p.E318K	WT		
Melanoma Risk[a]				
All patients	9 (1.9)	453 (98.1)	3.3 (1.43-7.43)	<.01
MPM	7 (2.6)	264 (97.4)	4.5 (1.83-11.01)	<.01
Phenotypic Features				
All MM patients				
>200 Nevi	4 (44.4)	28 (8.7)	8.4 (2.14-33.19)	<.01
Missing	0	130		
Fair skin	6 (66.7)	207 (57.3)	1.49 (0.37-6.04)	.74
Missing	0	92		
Non-blue eyes	8 (88.9)	264 (78.3)	2.21 (0.27-17.98)	.69
Missing	0	116		
Dark hair	6 (66.7)	232 (68.6)	0.90 (0.22-3.72)	>.99
Missing	0	115		
MPM				
>200 Nevi	4 (57.1)	19 (9.7)	12.4 (2.58-59.7)	<.01
Missing	0	68		
Fair skin	5 (71.4)	121 (58.5)	1.8 (0.34-9.37)	.70
Missing	0	57		
Non-blue eyes	6 (85.7)	140 (73.7)	2.1 (0.25-18.24)	.68
Missing	0	74		
Dark hair	5 (71.4)	126 (65.6)	1.3 (0.25-6.93)	>.99
Missing	0	72		
Familial history of pancreatic cancer				
Presence	1 (11.1)	15 (4.29)	2.9 (0.34-24.49)	.33
Missing	0	93		

Abbreviations: MM, malignant melanoma; MPM, multiple primary melanoma; OR, odds ratio; WT, wild-type.

[a] Data from 1659 French, 546 Italian, and 499 Spanish cancer-free controls were used. The *MITF* p.E318K frequency in the controls was 0.6% (16 of 2704).

95% CIs were calculated. The 2-sided Fisher exact test was used to look for statistical significance in proportion comparison. Age of onset was tested using an unpaired, 2-tailed *t* test. Breslow thickness was evaluated using the Mann-Whitney test. The results were considered statistically significant at $P < .05$. Statistical analyses were conducted using SPSS, version 17.0 (SPSS Inc). Data analysis was conducted from September 1 to November 30, 2014.

Results

CDKN2A gene, coding for the p16INK4A and p14ARF proteins, is a high-penetrance susceptibility in melanoma. Therefore, we calculated the prevalence of the *MITF* p.E318K variant in all 531 patients separated according to p16INK4A status (wild-type or mutated): 462 patients with wild-type p16INK4A and 69 patients with mutated p16INK4A. Among these, the prevalence of the *MITF* p.E318K variant was 1.9% (9 of 462) in all melanoma patients with wild-type p16INK4A, 2.6% (7 of 271) in those with MPM, and 2.9% (2 of 69) in the probands of families bearing a mutation in p16INK4A. All individuals with *MITF* p.E318K carried the variant in heterozygosis. The prevalence of the variant in a Spanish cancer-free population was 0.4% (2 of 499), with no statistically significant difference with French or Italian controls ($P = .54$).[14,16] With results reported as OR (95% CI), the *MITF* variant p.E318K increased the risk of developing melanoma in all melanoma patients with wild-type p16INK4A (3.3 [1.43-7.43]; $P < .01$) and in those with MPM (4.5 [1.83-11.01]; $P < .01$), using the Mediterranean controls (**Table 1**). When calculating the OR using, as a control population, the European non-Finnish population from the ExAC/Broad Institute exome database (http://exac.broadinstitute.org/, which gives an *MITF* p.E318K allele frequency of 0. 21% [140 *MITF* p.E318K alleles for 66732 total allele number]), the risk of developing melanoma in *MITF* p.E318K carriers was 4.7 (2.40-9.35; $P < .01$) and the risk of developing MPM was 6.3 (2.93-13.63; $P < .01$). We assessed the association between clinical and phenotypic features and the presence of p.E318K. The presence of the variant was associated with a very high nevi count (>200) in all patients with wild-type p16INK4A (8.4 [2.14-33.19]; $P < .01$) and in those with MPM (12.4 [2.58-59.7]; $P < .01$). We did not find any association with other phenotypical characteristics, family history of pancreatic cancer (Table 1), or clinicohistopathologic characteristics of tumors (**Table 2** and eTable 1 in the Supplement).

Table 3 reports the detailed clinical, phenotypic, and genetic characteristics of all patients with melanoma carrying *MITF* p.E318K. Patient M0881-01, with 3 previous melanomas, developed a fast-growing nodular melanoma that had not been present in a visit 2 months earlier that included total body photography. The patient detected a

Table 2. Clinicohistopathologic Features According to the Presence of *MITF* p.E318K in Patients With p16INK4A Wild-Type

Characteristic	*MITF* p.E318K	WT	OR (95% CI)	*P* Value
All Patients (N = 462)[a]				
Melanoma subtype, No. (%)[b]				
SSM	8 (100)	295 (86.3)	NA	.60
NM	1 (12.5)	35 (10.2)	1.25 (0.15-10.48)	.58
LMM	1 (12.5)	42 (12.3)	1.02 (0.12-8.50)	>.99
ALM	1 (12.5)	11 (3.2)	4.30 (0).49-38.02)	.25
Other	0	6 (1.8)	NA	>.99
Missing	1 (11.1)	111 (24.5)	NA	
Melanoma location, No. (%)				
Head or neck	2 (22.2)	54 (14.3)	1.71 (0.35-8.47)	.62
Trunk	5 (55.6)	198 (52.4)	1.13 (0.30-4.30)	>.99
Extremity	7 (77.8)	237 (62.7)	2.08 (0.43-10.16)	.50
Other	0	3 (0.8)	NA	>.99
Missing	0	75 (16.6)	NA	
MPM (n = 271)				
Melanoma subtype				
SSM	7 (100)	197 (92.1)	NA	.66
NM	1 (14.3)	22 (10.3)	1.5 (0.17-12.64)	.54
LMM	1 (14.3)	32 (15.0)	0.9 (0.11-8.14)	>.99
ALM	1 (14.3)	7 (3.3)	4.9 (0.52-46.62)	.23
Other	0	3 (1.4)	NA	>.99
Missing	0	51 (19.3)	NA	
Melanoma location				
Head or neck	1 (14.3)	38 (16.7)	0.8 (0.10-7.12)	>.99
Trunk	5 (71.4)	173 (75.9)	0.8 (0.15-4.21)	>.99
Extremity	6 (85.7)	129 (56.6)	4.6 (0.54-38.87)	.24
Other	0	2 (0.9)	NA	>.99
Missing	0	37 (14.0)	NA	
≥3 Primaries	2 (28.6)	51 (23.9)	1.2 (0.24-5.92)	.83
Breslow thickness, No. (median) [SD][c]				
All melanomas	12 (0.90) [1.54]	360 (0.76) [1.82]	NA	.43
First melanoma	5 (1.00) [2.26]	230 (0.89) [2.17]	NA	.33
Missing	1	119	NA	
Age at diagnosis of first melanoma, mean (SD), y [No.]	44.22 (16.60) [9]	46.83 (16.38) [406]	NA	.64
Missing, No.	0	47	NA	

Abbreviations: ALM, acral lentiginous melanoma; LMM, lentigo malignant melanoma; MPM, multiple primary melanoma; NA, not applicable; NM, nodular melanoma; SSM, superficial spread melanoma; WT, wild-type.

[a] There was a total of 462 patients. The number indicates the total number of primary melanomas diagnosed with the subtype or location indicated in each row, within each group of patients (*MITF* p.E318K carriers or patients with the WT *MITF*).

[b] Data on subtypes were determined based on the number of patients with at least 1 tumor of this subtype or location. Data on missing information refers to the total number of patients with missing information.

[c] In situ melanomas were not considered when calculating the Breslow median thickness (millimeters).

fast-growing hypopigmented lesion on the elbow that arose 3 weeks before an urgent evaluation at our unit (**Figure 1**). With dermoscopy, the lesion showed an unspecific pattern, with asymmetry in the distribution of colors and structures, the presence of blue-gray color, and milky-red areas with some vessels. In confocal microscopy, the lesion showed some bright, large, round cells in the upper epidermis around a central ulceration; in the dermoepidermal junction, papilla were not well demarked and not visible in some areas with dermal nests of noncohesive bright cells with large nuclei, highly suggestive of melanoma. The lesion was excised the same day with the final diagnosis of nodular melanoma with Breslow thickness of 1.3 mm, ulceration, 5 mitoses/mm^2, and epithelioid cells. Wide excision was performed and sentinel lymph node biopsy identified 3 negative sentinel lymph nodes.

Patient M1340-01, in follow-up for recurrent lentigo maligna melanoma, had also developed a fast-growing melanoma, in this case amelanotic, that had not been present in a visit 4 months earlier. Under dermoscopy, the lesion also showed an unspecific pattern with remnants of pigmentation, the presence of doted and linear irregular vessels, and short white streaks. The lesion was excised and the diagnosis was superficial-spreading melanoma in a vertical growth phase with a Breslow thickness of 1.65 mm, lack of ulceration, 3 mitoses/mm^2, and fusocellular morphology. Wide excision was performed, and sentinel lymph node biopsy identified 3 negative sentinel lymph nodes.

Patient M3879-01 belonged to a family with 2 cases of melanoma; thus, we assessed whether the other patient carried the variant. In this case, *MITF* p.E318K did not segregate with melanoma; however, the carrier was younger at the time of diag-

Table 3. Clinical, Phenotypic, and Genetic Features of the Spanish *MITF* p.E318K Carriers

Identification, Patient	No. of Melanomas	Sex/Age of Onset, y	Hair/Eye Color	Phototype[a]	Nevi Count	Other Tumors	*CDKN2A* Mutations		MC1R Missense Variants[b]
							p16INK4A	p14ARF	
M0109-01	NA	M/NA	NA/NA	NA	NA	NA	p.D84Y	p.R98L	R142H
M0881-01	4	M/40s	Brown/brown	II	>200	BCC	No	p.G32R	I155T
M1340-01	2	M/60s	Brown/blue	II	<50	BCC and RCC	No	No	R151C and V92M
M1545-01	2	F/40s	Blond/green	II	>200	BCC	No	No	V60L
M1569-01	4	M/20s	Blond/green	II	100-200	No	No	No	WT
M3824-01	2	F/70s	Brown/brown	II	<50	No	No	No	WT
M3879-01	2	F/30s	Brown/brown	III	>200	No	No	No	WT
M3879-03	0	F/NA	Black/brown	II	>200	No	No	No	V60L
M3879-09	0	F/NA	Brown/brown	II	100-200	No	No	No	WT
M4182-01	2	M/20s	Brown/brown	III	>200	No	No	No	R160W
M4619-01	1	M/30s	Blond/green	II	100-200	No	No	No	V92M
M4713-01	1	M/40s	Brown/brown	III	50-100	No	No	No	V92M
M4999-01	1	F/40s	Blond/green	II	>200	No	p.A127S	No	R163Q

Abbreviations: BCC, cutaneous basal cell carcinoma; NA, not available; RCC, renal cell carcinoma; WT, wild-type.

[a] The phototype is indicated using the Fitzpatrick Scale.

[b] All variants were detected in heterozygosis.

nosis (30s vs 70s). We also detected 2 healthy individuals carrying the variant in this family: M3879-03 and M3879-09 (phenotypic features of those 2 individuals are also recorded in Table 3). The nevi from carriers followed a reticular pattern and were dark brown (**Figure 2**).

Discussion

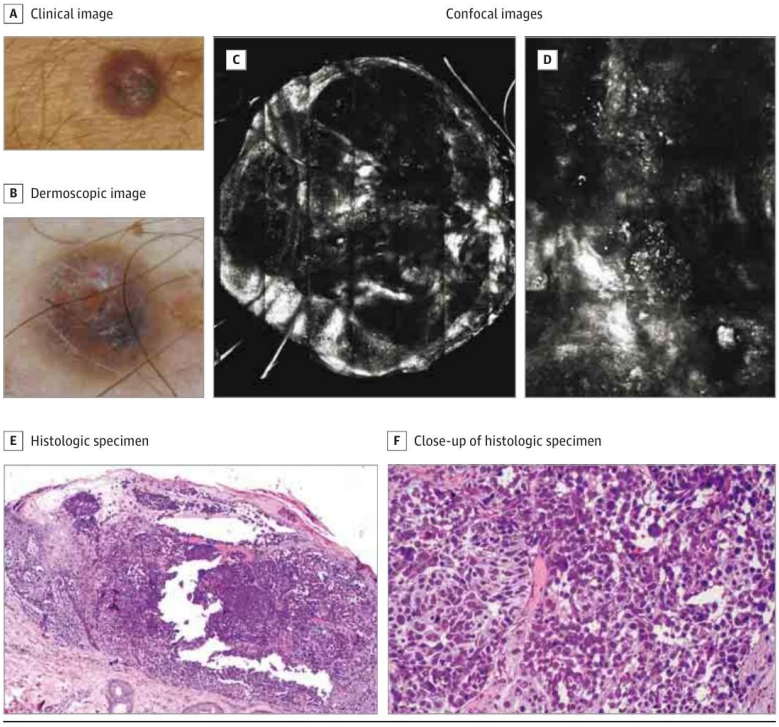
In this study, we analyzed the prevalence of *MITF* p.E318K in Spanish patients with melanoma. To our knowledge, this is the first study in which a set of individuals bearing mutations in *CDKN2A* affecting p16INK4A was also tested for *MITF* p.E318K. We detected a prevalence of 1.9% of the variant in all patients with wild-type p16INK4A, which was higher in the MPM subgroup (2.6%), and a similar prevalence was found in the set of patients with the p16INK4A mutation (2.9%). Previous studies[14-17,19] reported that *MITF* p.E318K increases the risk of developing melanoma (eTable 2 in the Supplement). We also detected this association in our set of patients. In addition to reporting increased melanoma risk, Yokoyama and colleagues[15] stated that the presence of this variant was associated with a high nevi count in an Australian and UK population. We also found that *MITF* p.E318K is associated with a very high nevi count (>200 nevi) in a Mediterranean population. These findings suggest that *MITF* may be involved in nevogenesis. Twin studies[20-22]have revealed evidence that the nevi count is genetically determined, with an additive genetic variance of 36% to 84%, increasing with age. The nevi count is a polygenic trait determined by multiple alleles.[23-27] The present study and the results in Australian and UK populations indicate that *MITF* p.E318K should be included in the set of known genes involved in this phenotypical trait.

To our knowledge, only one study[19] has described the phenotypical features and dermoscopic pattern of nevi from *MITF* p.E318K carriers in Australian patients. The investigators observed that these carriers had pink or light brown nevi, suggesting that *MITF* p.E318K could modulate nevi pigmentation. In contrast, in our study, the nevi were dark brown, indicating that other genes may be involved in this feature. The *MC1R* gene may modulate the pigmentation of the nevi since melanomas from carriers of RHC variants are less pigmented.[28] Otherwise, in the Australian study[19] and ours, the dermoscopic pattern of nevi present in *MITF* p.E318K carriers was predominantly reticular. These findings are suggestive of photoinduced nevogenesis.[29]

Sturm and colleagues[19] noticed a high incidence of amelanotic melanoma within *MITF* p.E318K carriers. One of our patients (M1340-01) also developed this type of melanoma. The patient carried one *MC1R* RHC variant: p.R151C. However, although our findings support the hypothesis that a genetic interaction between *MC1R* RHC and *MITF* p.E318K could increase the risk of developing amelanotic melanomas, it has been reported[17] that the interaction of *MITF* and *MC1R* variants is not associated with melanoma pigmentation.

Ghiorzo and colleagues[16] found an association between *MITF* p.E318K and the presence of nodular melanomas. We did not detect a significant association with any clinicohistopathologic features, probably because the sample size lacked the power to detect these possible associations. However, we noted 2 fast-growing melanomas in 2 *MITF* p.E318K carriers who were receiving dermatologic surveillance owing to a previous melanoma diagnosis. Dermatologic digital follow-up has been demonstrated[30,31] to be relevant for detecting melanomas at early stages with a low rate of excisions in patients at high risk to develop melanoma. During 10 years of dermatologic surveillance of patients at high-risk of melanoma in our melanoma unit (from January 1, 1999, to December 31, 2008), 98 new melanomas were diagnosed in these patients; 54% were in situ melanoma and 46% were invasive melanoma. Among the invasive melanomas diagnosed, none was more than 1-mm Breslow thickness and no melanomas behaved as fast-

Figure 1. Melanoma



A fast-growing melanoma developed within 3 weeks and was the fourth to occur in patient M0881-01. Clinical picture of a 4-mm-diameter nodular lesion located on the elbow (A); dermoscopic image of the lesion showing hypopigmentation, asymmetry, unspecific pattern, atypical vessels, and blue-whitish veil (B). Under confocal microscopy, the lesion shows an ulcerated central area (C) with atypical nests in upper dermis with bright roundish nucleated cells in noncohesive nests (D). Histopathologic examination shows an ulcerated nodular melanoma (hematoxylin-eosin, original magnification ×2) (E) and nests of atypical cells and presence of mitosis (hematoxylin-eosin, original magnification ×10) (F).

Figure 2. Nevi

The back of patient M3879-01 with 2 previous melanomas and more than 200 nevi. Six dermoscopic images show the predominant pattern, reticulated dark brown.

growing melanomas.[31,32] Until now in our melanoma unit, the only 2 fast-growing melanomas identified by dermatologic digital follow-up in individuals at high risk of melanoma were in *MITF* p.E318K carriers. Fast-growing melanomas are defined by having a growth rate of greater than 0.4 mm per month; in general, the melanoma growth rate is approximately 0.1 mm per month, and slow-growing melanomas usually have a growth rate of 0.01 mm per month.[33] Furthermore, a high growth rate is

associated with a worse prognosis in melanoma; thus, strategies for early detection of fast-growing melanomas are necessary.[34] Although further studies should address the role of *MITF* p.E318K in fast-growing melanoma, the carriers of *MITF* p.E318K should be encouraged to perform monthly total-body self-examination of the skin and receive fast-track, urgent dermatologic visits if any new lesion appears.

Genetic counseling is increasingly being offered to patients with sporadic MPM or familial melanoma and/or to their healthy relatives.[35] The genetic counseling in melanoma is focused on the screening of high-penetrance genes such as *CDKN2A*. Although *MITF* p.E318K is a moderate melanoma risk allele, it also increases the risk of developing RCC and pancreatic cancer. In our set of carriers, we observed that 42.9% (3 of 7) of the patients carrying *MITF* p.E318K developed cutaneous basal cell carcinoma, which is similar to previous data reported in wild-type *CDKN2A* MPM.[36] We did not detect any association with pancreatic cancer, probably owing to the small number of carriers in the study. Reinforcing their predisposition to develop RCC, 14.3% (1 of 7) carriers had developed this kind of tumor. Thus, if genetic counseling included *MITF* p.E318K genetic testing, individuals carrying the *MITF* p.E318K variant could benefit from being included both in melanoma and RCC prevention/surveillance programs. Furthermore, the detection of *MITF* p.E318K may identify patients at risk of developing fast-growing melanomas. We have observed a similar prevalence of *MITF* p.E318K in cases with germline *CDKN2A* mutations as in patients with wild-type. However, further studies should be performed to assess the role of *MITF* p.E318K as a possible modulator of the effect of *CDKN2A* mutations. This result suggests that individuals with a mutation in *CDKN2A* might also be included in *MITF* p.E318K screening as the identification of this variant allows for better characterization of the risk in the family and to adapt the cancer surveillance programs accordingly.

Patients with *MITF* p.E318K should be encouraged to follow melanoma prevention programs, which include sun protection strategies, monthly self-examination of the skin, and dermatologic surveillance. Because *MITF* p.E318K has been associated with RCC,[14] the use of renal ultrasonography as a safe and low-cost screening technique to detect the presence of kidney tumors should be considered. Future studies should explore the cost-efficacy and acceptance of this screening technique in *MITF* p.E318K carriers. It would be important to recommend that carriers avoid smoking and control their weight since smoking and obesity are important risk factors for both RCC and pancreatic cancer.[37,38] Moreover, detection of *MITF* p.E318K in a patient leads to the possibility of extending genetic testing to other relatives, and positive cases should be encouraged to follow the same preventive measures.

Conclusions

Based on the results of this study, *MITF* (and *MC1R*) should be added to *CDKN2A/CDK4* genetic testing based on published international recommendations for countries with low and high sun exposure.[39] Genotyping for *MITF* (and *MC1R*) could be added to predictive testing for all relatives in *CDKN2A*-positive families.

ARTICLE INFORMATION

Accepted for Publication: September 18, 2015.

Published Online: December 9, 2015. doi:10.1001/jamadermatol.2015.4356.

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Author Contributions: Drs Puig and Potrony had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
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Statistical analysis: Potrony.
Obtained funding: Malvehy, Puig.
Study supervision: Puig.

Conflict of Interest Disclosures: None reported.

Funding/Support: The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants 09/01393 and 12/00840; Centro de Investigación Biomédica en Redde Enfermedades Raras of the Instituto de Salud Carlos III, Spain; Agència de Gestió d'Ajuts Universitaris i de Recerca (AGUAR) 2009 SGR 1337 and AGAUR 2014_SGR_603 of the Catalan Government, Spain; European Commission under the Sixth Framework Programme, contract No. LSHC-CT-2006-018702 (GenoMEL) and by the European Commission under the Seventh Framework Programme, Diagnoptics; the National Cancer Institute of the National Institutes of Health (CA83115); and a grant from Fundació La Marató de TV3, 201331-30, Catalonia, Spain. Ms Potrony is the recipient of a PhD fellowship from Instituto de Salud Carlos III, Spain.

Role of the Funder/Sponsor: The funding organizations had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Additional Contributions: The work was carried out at the Esther Koplowitz Center in Barcelona. The Spanish National DNA Bank performed sample and data procurement of the control population. We thank the nurses of the melanoma unit: Maria Eugenia Moliner, BSs; Pablo Iglesias, BSs; and Daniel Gabriel, BSs (Melanoma Unit, Dermatology Department, Hospital Clínic de Barcelona); they received no compensation other than their salary. We also thank all of the residents and fellows who contributed to the tasks in the melanoma unit as well as the patients and their families. We thank the patients for granting permission to publish this information.

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Supplementary Online Content

Potrony M, Puig-Butille JA, Aguilera P, et al. Prevalence of *MITF* p.E318K in patients with melanoma independent of the presence of *CDKN2A* causative mutations. *JAMA Dermatol*. Published online December 9, 2015. doi:10.1001/jamadermatol.2015.4356.

eTable 1. Primary Melanoma Characteristics From Patients With *MITF* p.E318K

eTable 2. Summary of the *MITF* p.E318K Reports in Melanoma Patients

This supplementary material has been provided by the authors to give readers additional information about their work.

eTable 1. Primary Melanoma Characteristics From Patients With *MITF* p.E318K

Patient ID	Family ID	MM	Year	Location	Clark level	Breslow thickness	Subtype
M0109-01	M0109	NA	NA	NA	NA	NA	NA
M0881-01	M0881	1st	1995	Trunk	IV	6.00	SSM
		2nd	2001	Trunk	III	0.50	SSM
		3rd	2004	Trunk	III	0.80	SSM
		4th	2013	Upper extremity	IV	1.30	NM
M1340-01	M1340	1st	2003	Head	I	in situ	LMM
		2nd	2006	Trunk	III	1.60	SSM
M1545-01	M1545	1st	2005	Lower extremity	II	0.50	SSM
		2nd	2005	Upper extremity	II	NA	SSM
M1569-01	M1569	1st	2005	Trunk	I	in situ	SSM
		2nd	2005	Lower extremity	II	0.90	SSM
		3rd	2012	Lower extremity	I	in situ	SSM
		4th	2013	Trunk	NA	NA	SSM
M3824-01	M3824	1st	2009	Lower extremity	IV	2.20	SSM
		2nd	2012	Foot	I	in situ	ALM
M3879-01	M3879	1st	2003	Lower extremity	I	in situ	SSM
		2nd	2003	Trunk	II	0.60	SSM
M4182-01	M4182	1st	1997	Trunk	NA	0.90	SSM
		2nd	2004	Lower extremity	NA	0.35	SSM
M4619-01	M4619	1st	1995	Trunk	NA	NA	NA
M4713-01	M4713	1st	2012	Scalp	IV	1.00	SSM
M4999-01	M4999	1st	2014	Lower extremity	II	0.60	SSM

MM: melanoma; NM: nodular melanoma; SSM: Superficial Spread melanoma, LMM: Lentigo malignant melanoma; ALM: Acral Lentiginous melanoma.
Breslow thickness is expressed in millimeters.

eTable 2. Summary of the *MITF* p.E318K Reports in Melanoma Patients

Study	Patients and origin	N cases	Melanoma Risk	Age of onset	High nevi count	non-blue eyes	Fair skin color	Dark hair color	Renal cancer	pancreatic cancer	Nodular melanoma
			OR (95% CI)		OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Yokoyama et al. 2011	Australia + UK										
	All patients	3988	**2.19 (1.41–3.45)**		**2.5 (1.42–4.55)**	**2.0 (1.11–3.81)**	N.S.	N.S.			
	Australia										
	All patients	2059	**2.33 (1.21–4.70)**	N.S.							
	familial MM	575	**2.95 (1.23–6.92)**								
	MPM	270	**4.22 (1.52–10.91)**								
	familial MPM	105	**8.37 (2.58–23.80)**								
	UK										
	All patients	1929	**2.09 (1.14–3.94)**	N.S.							
	familial MM	110	N.S.								
	MPM	89	N.S.								
Bertolotto et al. 2011	France, Italy and Spain										
	All patients	603	**4.78 (2.05–11.75)**						**5.2 (1.37–16.87)**[†]		
	MPM	288	**7.79 (3.12-20.04)**								
Ghiorzo et al.	Italy										

2013	All patients	679	**2.85 (1.31-6.18)**						**8.0 (1.62-39.46)**	**30.9 (6.85-138.9)**	**4.48 (1.39-14.43)**
	Familial single MM	129	N.S.								
	Sporadic MPM	52	**6.40 (1.43-28.58)**								
Sturm et al. 2013	Australia	6	-		increased nevus count		All patients				no association
Berwick et al. 2014	Australia, Italy, Canada, USA										
	MPM	1194	**1.7 (1.1-2.6)***		N.S.	**2.1 (1.2-3.8)**	**2.2 (1.2-3.8)**	**3.8 (1.5-9.6)**			
Present study	Spain										
	All patients	511	**2.9 (1.24-6.69)**	N.S.	**9.0 (2.30-35.45)**	N.S.	N.S.	N.S.	1/9 carriers	N.S.	N.S.
	MPM	271	**4.2 (1.67-10.44)**		**12.4 (2.58-59.7)**	N.S.	N.S.	N.S.			

MM: melanoma, MPM: multiple primary melanoma, N.S.: Non significant, OR: Odds Ratio, 95% CI: 95% Confidence Interval

*Sporadic single melanoma patients were used as controls

†Renal cancer risk was evaluated in an independent cohort of 829 patients with renal cancer.

ARTICLE 3

POT1 germline mutations but not *TERT* promoter mutations are implicated in melanoma susceptibility in a large cohort of Spanish melanoma families.

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Manuscript under review in British Journal of Dermatology (first submission 11/05/2018)

Aim

To evaluate the prevalence of germline mutations in *POT1* and the *TERT* promoter in a set of Spanish patients from melanoma-prone families or a history of multiple primary melanomas

Patients

328 melanoma patients visited at the Melanoma Unit – Hospital Clínic of Barcelona (January 1994 – June 2015):

- 228 probands from *CDKN2A* wild-type families (*POT1* and *TERT* promoter analysis)
- Additionally, for *TERT* promoter study only we included
  - 70 *CDKN2A* wild-type sporadic MPM
  - 30 probands from *CDKN2A*-positive melanoma-prone families

Methods

POT1 molecular screening was performed by WES analysis in 82 samples and Fluidigm unidirectional sequencing protocol in the 146 remaining. Variants detected by those NGS techniques were sequenced using Sanger sequencing protocol for confirmation.

When a pathogenic variant was detected, the genetic study was extended to the rest of the family for segregation analysis (if DNA for other members was available).

When a variant was predicted to affect splicing, studies at the mRNA level where designed.

TERT promoter, including the rs2853669 polymorphism position, was sequenced using Sanger sequencing protocol.

Descriptive study and statistical association analysis for rs2853669.

Variables included in description

Presence of *POT1* variants

rs2853669 genotype

Family and personal history of other cancers in families with variants detected.

Main results

POT1 probably pathogenic variants were detected in 1.75% of the families tested. Four mutations were detected:

- c.233T>C (p.Ile78Thr), a missense variant
- c.1030G>T (p.Glu344*), a nonsense variant
- c.255G>A (r.125_255del), confirmed disrupted *POT1* mRNA splicing
- c.1792G>A (r.1791_1792insAGTA, p.Asp598Serfs*22), confirmed disrupted *POT1* mRNA splicing.

Thyroid cancer and goiter were developed by mutation carriers, reinforcing the possible role of *POT1* in the development of thyroid malignancies.

No mutations were detected in *TERT* promoter, independent of the *CDKN2A* germline status in familial melanoma. A variant of unknown significance (c.-125C>A) was identified in a sporadic MPM patient.

Title: *POT1* germline mutations but not *TERT* promoter mutations are implicated in melanoma susceptibility in a large cohort of Spanish melanoma families.

Running head: *POT1* and *TERT* promoter molecular screening in Spanish melanoma families

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Funding sources:

The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants PI15/00716 and PI15/00956; CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain, co-financed by European Development Regional Fund "A way to achieve Europe" ERDF; AGAUR 2017_SGR_1134 of the Catalan Government, Spain; European Commission under the 6th Framework Programme, Contract No. LSHC-CT-2006-018702 (GenoMEL) and by the European Commission under the 7th Framework Programme, Diagnoptics; The National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115); a grant from "Fundació La Marató de TV3"

201331-30, Catalonia, Spain; a grant from "Fundación Científica de la Asociación Española Contra el Cáncer" GCB15152978SOEN, Spain, and CERCA Programme / Generalitat de Catalunya. Part of the work was carried out at the Esther Koplowitz Center, Barcelona.

The whole exome sequencing analysis was in part supported by the intramural research program of the National Institutes of Health, National Cancer Institute, Division of Cancer Epidemiology and Genetics.

DJA is funded by Cancer Research UK, The Wellcome Trust and the ERC Synergy Programme.

C. D. Robles-Espinoza's work is funded by the Wellcome Trust (Seed Award Ref. 204562/Z/16/Z) and Universidad Nacional Autónoma de México (PAPIIT award ref. IA200318).

Miriam Potrony is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain.

Role of the Sponsors: The sponsors had no role in the design and conduct of the study; in the collection, analysis and interpretation of data; or in the preparation, review, or approval of the manuscript.

Conflict of Interest Disclosure: None Declared.

Bulleted statements:

What's already known about this topic?

- Telomere-related genes germline mutations predispose to familial melanoma.
- The prevalence of germline mutations in telomere-related genes has not been widely studied in Iberian-descent melanoma families.

What does this study add?

- This study evaluates for the first time the prevalence of *POT1* and *TERT* promoter mutations in a Hospital-based series of 228 *CDKN2A*-negative melanoma families from Barcelona, Spain.
- We have identified *POT1* mutations in 1.75% families, but no *TERT* promoter mutations in our series.
- These results will facilitate genetic counseling and screening of melanoma families.

SUMMARY

Background: Germline mutations in telomere-related genes such as *POT1* and the *TERT* promoter predispose to familial melanoma.

Objective: To evaluate the prevalence of germline mutations in the *POT1* gene and in the *TERT* promoter in a large cohort of Spanish melanoma-prone families (at least two affected individuals in first- or second-degree relatives).

Methods: The *POT1* gene and the *TERT* promoter were screened in one case/family from 228 *CDKN2A* wild-type melanoma-prone families. *TERT* promoter sequencing was extended to 70 sporadic multiple primary melanoma patients (MPM) with a family history of other cancers and to 30 *CDKN2A* mutated families.

Results: We identified four families with potentially pathogenic *POT1* germline mutations: a missense variant c.233T>C (p.Ile78Thr), a nonsense variant c.1030G>T (p.Glu344*), and two variants c.255G>A (r.125_255del) and c.1792G>A (r.1791_1792insAGTA, p.Asp598Serfs*22), which we confirmed disrupted *POT1* mRNA splicing. A *TERT* promoter variant of unknown significance (c.-125C>A) was detected in a MPM patient, but no germline mutations were detected in the *TERT* promoter in familial melanoma cases.

Conclusions: Overall, 1.75% of our *CDKN2A/CDK4*-wild type Spanish melanoma-prone families carry probably damaging mutations in *POT1*. The frequency of *TERT* promoter germline mutations in melanoma families in our population is extremely rare.

INTRODUCTION

Around 10% of melanoma cases report a family history of melanoma. In these families, genetic variants conferring susceptibility are inherited following an autosomal dominant pattern with incomplete penetrance. To date, *CDKN2A* is the main high-penetrance gene involved in melanoma susceptibility and around 20% to 40% of melanoma-prone families harbor *CDKN2A* mutations worldwide.[1,2] Mutation screening of *CDKN2A* and *CDK4* has been conducted in 330 Spanish melanoma-prone families from our group. Overall, *CDKN2A* mutations were present in 14% of families, While no *CDK4* positive families have been identified.[3]

Patients with multiple primary melanomas (MPM) but without a family history of melanoma may also have an increased susceptibility to develop melanoma and *CDKN2A* mutations have been detected in 8-10% of sporadic MPM patients.[4,5] Recent studies in melanoma-prone families using next-generation sequencing (NGS) approaches have identified other high penetrance melanoma susceptibility genes that play a role in telomere maintenance, such as *TERT*, *POT1*, *TERF2IP*, and *ACD*.[1] In particular, two independent studies have identified a germline mutation in the promoter of *TERT* (c.-57T>G) in two unrelated families of Northern-European ancestry.[6,7] This variant creates a new ETS transcription factor binding site in the *TERT* promoter and increases TERT expression.[7] More recently, rare *POT1* germline variants have been identified in *CDKN2A* wild-type melanoma-prone families from Northern- and Southern-European countries, USA, and Australia.[8,9] To date, these telomere-related genes have not been extensively studied in patients of Iberian descent.

Our aim was to evaluate the prevalence of germline mutations in *POT1* and the *TERT* promoter in a collection of Spanish patients from melanoma-prone families or a history of multiple primary melanoma.

PATIENTS AND METHODS

Families and Samples

POT1 and *TERT* promoter molecular screening was conducted in one melanoma patient with available DNA from each of 228 *CDKN2A* and *CDK4* wild-type families. The families had at least two melanoma cases in first- or second-degree relatives and were recruited at the Melanoma Unit of Hospital Clinic of Barcelona from 1994 to 2015. In addition, *TERT* molecular screening was performed in one melanoma patient from 30 *CDKN2A* mutation positive families and in 70 *CDKN2A* wild-type sporadic MPM patients with family history of other cancers diagnosed in first- or second-degree relatives. All patients signed written informed consent after reading and understanding the study protocol and agreeing to participate in the study. The study was approved by the ethics committee of the Hospital Clinic of Barcelona and the National Cancer Institute, NIH.

POT1 molecular screening

Whole exome sequencing was performed on 82 samples at the National Cancer Institute. Data analysis and extraction of *POT1* variants for these families was performed using the same methodology described in Shi et al.[8] The remaining 146 samples were processed at Sanger Institute. PCR primers were designed

against all annotated exons of *POT1* (ENST00000357628.7, NM_015450.2). Samples were PCR-amplified and individually barcoded following the Fluidigm unidirectional sequencing protocol.[10] Primers for PCR reactions were pooled up to 1151-plex per well in 9 pools. Generated libraries were sequenced, one per lane and producing 150-bp paired-end reads, on an Illumina MiSeq.[10]

Reads were aligned to the reference genome (GRCh37) using BWA mem. The 146 samples had at least 90% of the target bases in *POT1* of high quality (base quality >= 20, read mapping quality >=50). Variants were called with the GATK HaplotypeCaller, and quality filters were set as standard (minimum number of alternate bases 2, minimum read depth 2, minimum mapping quality for SNPs 10, window size for filtering adjacent gaps 3, and exclusion of SNPs within 10bp around a gap) minus the end-distance and stand bias filters. Variant consequences were predicted with the Ensembl Variant Effect predictor release 70.

We selected all frameshift, nonsense, missense and variants predicted to affect the splicing and with a frequency <0.01 in European non-Finnish ExAC samples. Specific PCR primers were designed to amplify the exons containing the selected variants (**Table S1**). PCR followed by Sanger sequencing was performed to validate the presence of the variants detected by NGS. PCR conditions were: denaturalization at 95°C 5 min, 10 cycles (95°C 1 min, 65°C–60°C 1 min, 72°C 1 min), followed by 25 cycles (95°C 1 min, 55°C 1 min, 72°C 1 min) and extension at 72°C (10 min). Sanger Sequencing was performed using universal M13 primers by GENEWIZ (Takeley, UK). Sequences were analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems, Germany). In those families with variant confirmation, the sequencing analysis was extended to other relatives if samples were available.

***POT1* mRNA studies**

To assess whether the two splice-site variants detected affected mRNA processing, two independent blood RNA samples from each carrier and blood RNA samples from seven healthy donors were obtained using the PAXgene Blood RNA Kit (PreAnalytiX®, Qiagen, Germany). The mRNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, US). Multiple PCRs were designed to amplify the mRNA region containing the coding region of exons 5 to 8, 16/17 to 19 and 17/18 to 19 of *POT1* (ENST00000357628.7, NM_015450.2). These regions were amplified from the cDNA from two independent blood samples from each patient and the healthy donors. The PCR products were assessed by electrophoresis. The different fragments amplified were isolated using PureLink Quick Gel Extraction kit (Invitrogen, CA, US) and sequenced using the corresponding primers described in **Table S1** by GENEWIZ (Takeley, UK).

***TERT* promoter molecular screening**

For *TERT* (ENST00000310581.9, NM_198253.2) promoter amplification, covering also the rs2853669 polymorphism position, specific PCR primers were designed based on the primers previously reported by Horn and colleagues (**Table S1**).[7] PCR followed by Sanger sequencing was performed as described above for the *POT1* molecular screening. PCR conditions were: denaturation at 95°C for 5 min, 10 cycles (95°C 1 min, 65°C 1 min, 72°C 1 min), followed by 25 cycles (95°C 1 min, 62°C 1 min, 72°C 1 min) and a terminal extension at 72°C (10 min).

RESULTS

POT1 molecular screening

Rare *POT1* variants were detected by NGS and confirmed by Sanger sequencing in 1.75% of pedigrees (4/228) (**Fig 1**). Co-segregation analysis of the detected variants was extended to the rest of the family where DNA samples were available. The c.233T>C (p.Ile78Thr), a missense variant predicted to be pathogenic, was identified in an affected individual from a 2-case family. This individual had two melanomas and thyroid cancer. The same variant was identified in other four unaffected individuals in the family, who were 55 years old or younger. The c.255G>A (p.Lys85Lys), a synonymous variant, predicted to affect splicing, was identified in two affected individuals from a 3-case family. One of the two affected individuals carrying the variant had two melanomas. In the family, there were also relatives with testicular cancer and a lung cancer, but we were unable to test this variant in these individuals. The c.1030G>T (p.Glu344*), a nonsense variant, was identified in the two affected individuals we could test in a 3-case family. The non-tested case in this family was an obligate carrier. The same variant was identified in a 41-year-old unaffected individual. Finally, the c.1792G>A (p.Asp598Asn), a missense variant, predicted to be pathogenic and predicted to affect splicing, was identified in a melanoma patient from a 2-case family. This individual also carried the *MITF* p.Glu318Lys variant. Another relative in the family had breast cancer but could not be tested.

To assess whether the variant c.255G>A alters splicing, we amplified by PCR and sequenced the cDNA region encompassing exons 5 to 8 and 6 to 8. Two different fragments amplified in both the carrier and 7 healthy individuals. Specific fragment sequencing confirmed exon 7 skipping of the shorter fragment, probably corresponding to an isoform that naturally excludes exon 7 (ENST00000393329.5, NM_001042594.1). We observed that the c.255G>A carrier had a higher proportion of fragments with exon 7 skipping. Furthermore, we sequenced the largest fragment, corresponding to the fragments including exon 7, using a specific internal primer that anneals exon 7. We observed only the wild-type allele, showing that the mutant allele only produces the shorter isoform (r.125_255del) (**Fig. 2**).

To assess whether the missense variant c.1792G>A affects splicing, we amplified by PCR and sequenced the cDNA region encompassing exons 16/17 to 19 and 17/18 to 19. A unique fragment was identified. The fragment sequencing confirmed that, at the RNA level, there exist an insertion of four nucleotides in the mutant allele (r.1791_1792insAGTA), incorporating part of the intron, which results in a reading frame alteration (p.Asp598Serfs*22). However, a residual presence of the transcript with the r.1792G>A (p.Asp598Asn) was also observed. This indicates that the nucleotide change is able to activate a cryptic splicing site, but a small amount of non-spliced altered mRNA is still produced (**Fig. 2**).

TERT promoter molecular screening

We sequenced the *TERT* promoter in one case/family from 202 *CDKN2A* wild-type melanoma-prone families (26 of our 228 families analyzed for *POT1* variants by NGS could not be analyzed due to exhausted or degraded DNA), 30 probands from *CDKN2A* mutation positive melanoma-prone families and 70 sporadic MPM with family history of other cancers were also analyzed. We detected a rare

germline variant of unknown significance (c.-125C>A) in a MPM patient with a family history of breast and colon cancer. No other mutations were detected in the abovementioned cases.

The distribution of the *TERT* promoter rs2853669 polymorphism was similar between MPM (49% TT, 47% TC, 4% CC) and familial cases (48% TT, 45% TC, 7% CC; p=0.778). There were no differences regarding the age of onset (p=0.445) or the number of melanoma primaries (p=0.857) according to the rs2853669 genotype (Table S2).

DISCUSSION

The present study describes the prevalence of mutations in the telomere-related genes *POT1* and the *TERT* promoter in a large set of Spanish melanoma-prone families. We found four probably pathogenic *POT1* variants and one variant of unknown significance in *TERT*.

TERT was the first gene involved in telomere maintenance that was identified as a high-penetrance susceptibility gene for familial melanoma.[6,7] We identified a rare variant (c.125C>T) of unknown significance in the *TERT* promoter in a sporadic MPM patient with family history of breast and colon cancers. We previously reported the patient when evaluating germline mutations in a set of sporadic cases.[11] No mutations were detected in *TERT* in the families irrespective of the *CDKN2A* mutational status. Although germline *TERT* promoter mutations are extremely rare in familial melanoma,[6,7,12] common variants in *TERT* increase melanoma risk with smaller effect size.[13] The *TERT* promoter rs2853669 polymorphism, which may affect cancer clinical outcomes,[14] was not associated with age of onset or presence of multiple primaries, and its prevalence was similar among the MPM and familial cases in our study. In previous studies, the c.-57T>G variant was identified in two large melanoma pedigrees. In one of them a MPM patient with bladder cancer and basal cell carcinomas was a carrier.[6] In the other study two melanoma patients also developed other cancers including ovary (both cases) and bladder, renal, breast and lung (just one case).[7] Unfortunately there are not enough cases with rare germline mutations in *TERT* promoter to assess if there are specific cancer types or traits that are enriched in subjects who carry *TERT* promoter mutations.

Unlike *TERT* promoter mutations, *POT1* germline mutations were present in a subset of Spanish melanoma-prone families. We have detected four probably pathogenic *POT1* germline mutations in 1.75% (4/228) of *CDKN2A/CDK4*-wild type families: the p.Ile78Thr variant, which was previously reported in a MPM patient[8] and three novel variants: a nonsense variant (p.Glu344*) and two variants affecting splicing of the *POT1* main transcript (c.255G>A, r.125_255del and c.1792G>A, r.1791_1792insAGTA). The prevalence of *POT1* mutations observed in this study is similar to the prevalence of the medium frequency melanoma risk variant p.Glu318Lys in the *MITF* gene.[15] A limitation of the present study is that segregation analysis in subjects with melanoma or other cancers could not be performed in some families due to the lack of DNA availability from some affected individuals. A caution note with regard to *POT1* variants pathogenic function or penetrance is necessary. In fact, in one family where we could test multiple individuals, we identified the p.Ile78Thr variant also in unaffected members, although these individuals were relatively young, thus still at risk of developing

melanoma. Moreover, one of the variants, p.Asp598Asn, was found in an affected individual also carrying a *MITF* pathogenic mutation. Further studies are necessary to estimate the penetrance and effect size of *POT1* variants on melanoma risk.

Clinical and phenotypic characterization of mutation carriers from families with germline alteration in melanoma susceptibility genes such as *CDKN2A* or *BAP1* have allowed to refine genetic counseling. *CDKN2A* germline mutations have been associated with the presence of atypical nevi,[16] early age at diagnosis or MPM.[3-5] Beyond melanoma, *CDKN2A* mutation carriers have increased risk of pancreatic cancer and other tobacco-related cancers, thus smoking avoidance can be recommended as a preventive strategy.[3,17] *BAP1* germline mutations confer risk to cutaneous and uveal melanoma, mesothelioma and renal tumors, thus ophthalmological examinations and screening for early mesothelioma or renal tumors detection can be implemented in carriers.[1]

Although the number of reported pedigrees with *POT1* mutations is limited, MPM patients are present in multiple pedigrees,[8,9,18] including the present study. Moreover, other cancer types occur in the pedigrees. A recent study has identified a *POT1* germline variant in a melanoma-prone family with multiple cases of thyroid cancer and goiter.[18] We identified two melanoma patients in different families carrying *POT1* mutations who also develop thyroid cancer or goiter, thus supporting a role for *POT1* variants in the predisposition of thyroid cancer and goiter. Robles-Espinoza and collaborators identified *POT1* mutations in individuals with cutaneous melanoma and breast or lung cancer. Other tumors were developed in those families but segregation could not be confirmed.[9] *POT1* germline mutations have also been identified in families with Li-Fraumeni-Like syndrome with cardiac angiosarcoma[19,20] and chronic lymphocytic leukemia.[21] Nevertheless, more studies should be performed to assess the role of *POT1* in the susceptibility to other cancers.

In conclusion, the analysis of telomere-related genes showed *POT1* rare variants in a subset of Spanish melanoma-prone families, while mutations in the *TERT* promoter were extremely rare. If extended to additional families and cancer types, these findings may have important implications for genetic counseling.

Acknowledgements:

Thanks to our patients and their families who are the main reason for our studies; to nurses from the Melanoma Unit of Hospital Clínic of Barcelona, Daniel Gabriel, Pablo Iglesias, Mireia Domínguez and Maria E Moliner for helping to collect patient data, and to Judit Mateu from the “Melanoma: image, genetics and immunology” group at IDIBAPS for her technical assistance.

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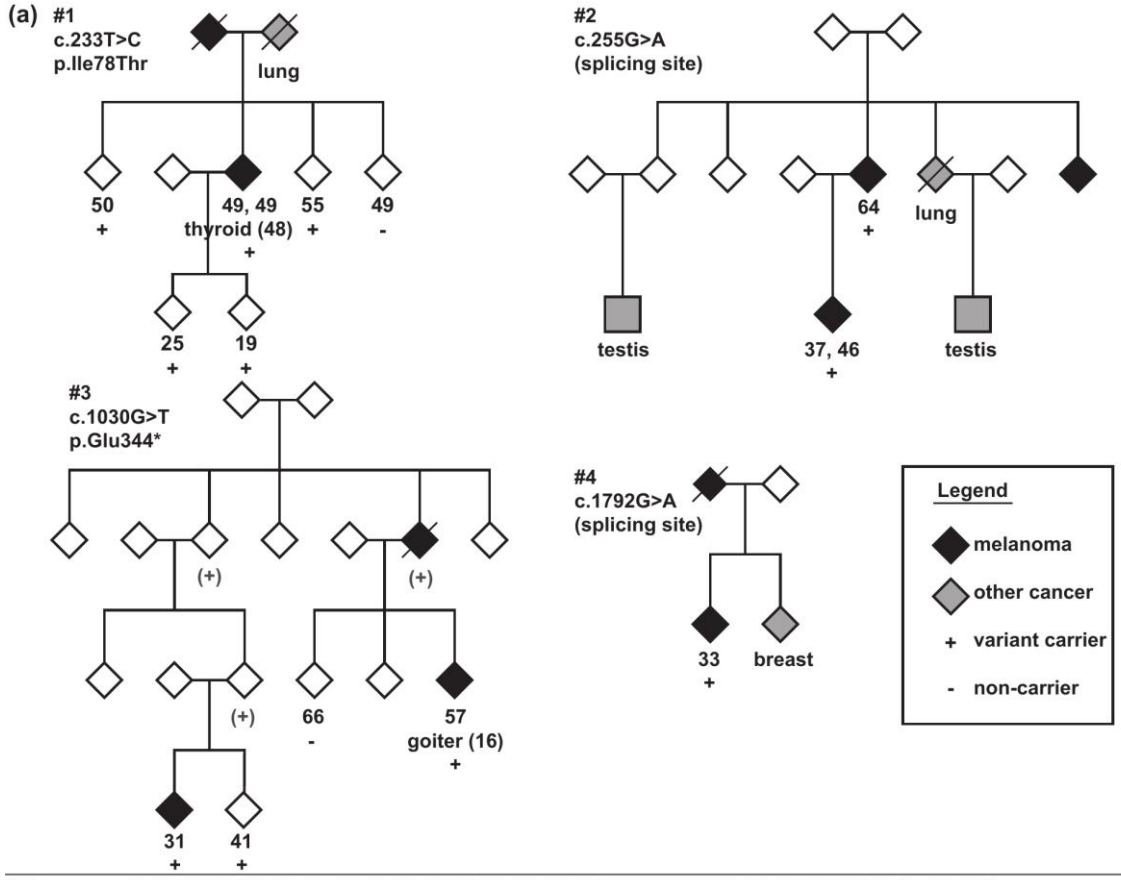
Figure 1. Pedigrees with *POT1* variants and variant information

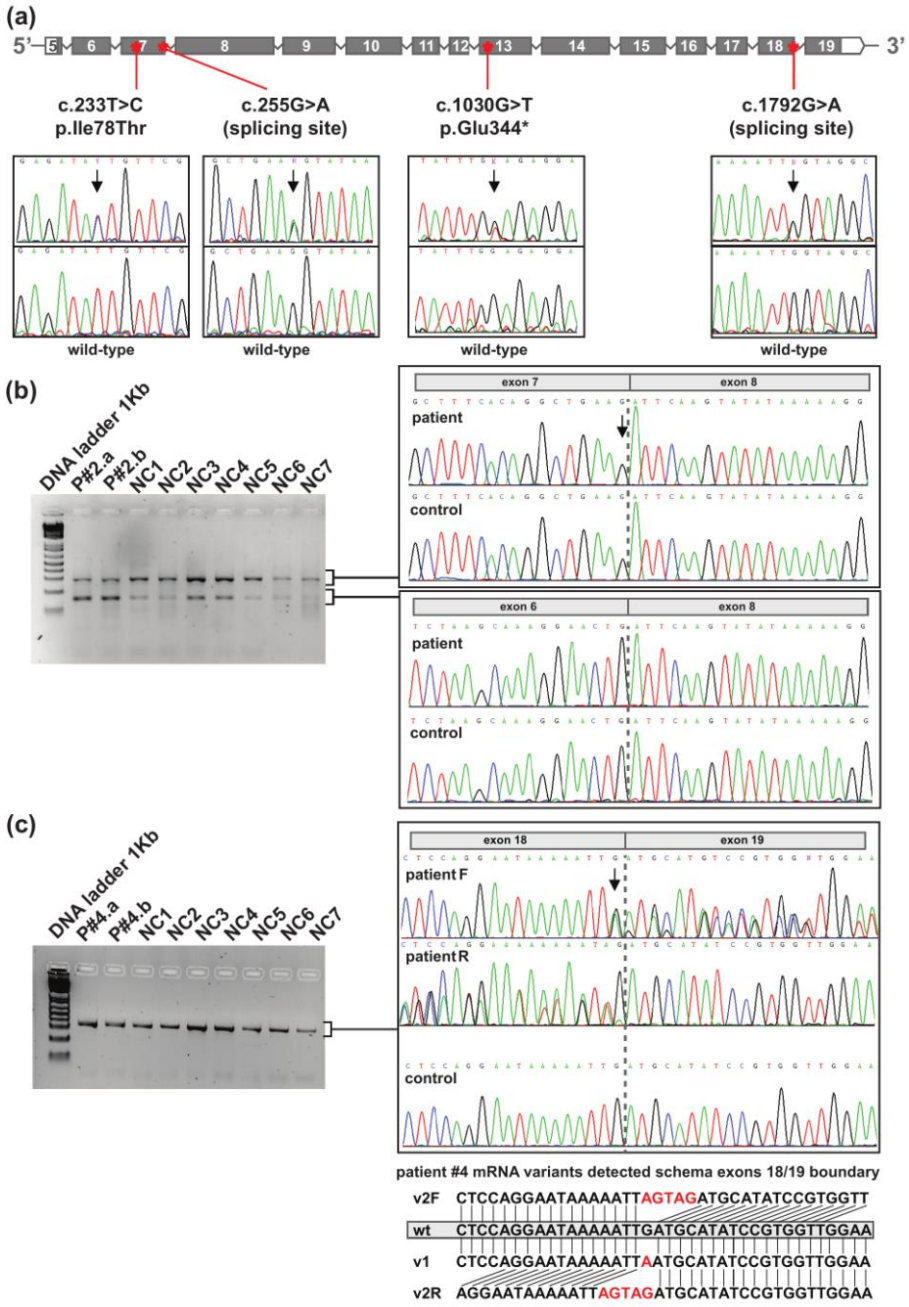


(b)

Pedigree	VARIANT (genomic position)	cDNA change	Type	ExAC E-NF	P	S	MT
#1	chr7:g.124510987A>G	c.233T>C	Missense	-	D	D	D
#2	chr7:g.124510965C>T	c.255G>A	Synonymous, affecting splicing site	1.53E-05	-	-	D
#3	chr7:g.124482994C>A	c.1030G>T	Nonsense	-	-	-	D
#4	chr7:g.124465306C>T	c.1792G>A	Missense, affecting splicing site	-	D	T	D

Pedigree diagrams from families with *POT1* germline rare variants. Gender has been hidden for family de-identification (except for individuals with gender-related cancer types). Below the symbol, age at diagnosis of each melanoma / age at blood sampling (for non-melanoma individuals), other cancer type / disease (age at diagnosis of other cancer/disease is indicated between parentheses) and presence "+" or absence "-" of the variant are listed. Non-tested obligated carriers are indicated as "(+)" in grey. b) Detailed information of the variant detected in each pedigree. ExAC E-NF: Variant frequency in European non-Finnish population from ExAC (http://exac.broadinstitute.org/) database. P: Polyphen2 functional prediction result (D=Probably damaging, P=Possibly damaging, B=Benign) (http://genetics.bwh.harvard.edu/pph2/). S: SIFT functional prediction result (D=Deleterious, T=Tolerated) (http://sift.jcvi.org/). MT: Mutation Taster functional prediction result (D=Disease Causing, N=Polymorphism,) (http://www.mutationtaster.org/)

Figure 2. *POT1* germline rare variants.



a) Location of the variants within the gene context (coding exons according to ENST00000357628.7 transcript) and Sanger sequencing confirmation of the variants detected. The arrows spot the position of the genomic DNA change. b) Gel electrophoresis of cDNA amplification corresponding to the coding region between exons 5 and 8 in Patient #2 (germline variant c.255G>A) and 7 non-carriers (NC). Sanger sequencing of the fragments detected. The dotted grey line marks the boundary between exons. c) Gel electrophoresis of cDNA amplification corresponding to the coding region between exons 17 and 19 in Patient #4 (germline variant c.1792G>A) and 7 non-carriers (NC). Sanger sequencing of the fragments detected. A schema of the different transcript alleles detected is shown (wt: wild-type r.1792G allele, v1: r.1792G>A allele and v2 (F=Forward; R=Reverse): r.1791_1792insAGTA).

Table S1. Primers list.

Gene	Primer	Region	Sequence	Product Length (bp)
POT1	Forward	Exon 7	TGTAAAACGACGGCCAGTGGTTTGGTGTTTTGAAGTAAGC	341
POT1	Reverse	Exon 7	CAGGAAACAGCTATGACCCTCCTGAAAAGCTTGCTGTC	
POT1	Forward	Exon 13	TGTAAAACGACGGCCAGTGGGAATAAAGACATAACCCTTCC	582
POT1	Reverse	Exon 13	CAGGAAACAGCTATGACCAGTCTGCCCAAATATATTCATCA	
POT1	Forward	Exon 18	TGTAAAACGACGGCCAGTTCATTATTAAATCAGAGCAATTTACTT	456
POT1	Reverse	Exon 18	CAGGAAACAGCTATGACCAAATCATTTGGAAGCAAAGC	
POT1	Forward	cDNA exons 5-8	CAATGTCTTTGGTTCCAGCA	293
POT1	Reverse	cDNA exons 5-8	GGTGATACCCTGAGTCTCCT	
POT1	Forward	cDNA exons 6-8	GTTGTGAAGTTCTTTAAGCCCC	235
POT1	Reverse	cDNA exons 6-8	ACGTCAAAGATGCAAAGCCA	
POT1	Forward	Exon 7 internal*	TGTAACTATTGTGGACCAGACA	-
POT1	Forward	cDNA exon 16/17	GCAGAAGCACTGGGTATTGT	369
POT1	Reverse	Exon 19	CAGGAAACAGCTATGACCGGTCAGGAAAAGAAGCTCAA	
POT1	Forward	cDNA exon 17/18	GGATTCTGACAAATTCTTCCA	297
POT1	Reverse	Exon 19	CAGGAAACAGCTATGACCGGTCAGGAAAAGAAGCTCAA	
TERT	Forward	promoter	TGTAAAACGACGGCCAGTCTGGCGTCCCTGCACCCTGG	474
TERT	Reverse	promoter	CAGGAAACAGCTATGACCACGAACGTCGCCAGCGGCAG	

*Only used for sequencing.

Table S2. Clinical characteristics according to *TERT* rs2853669 genotype

	TT	TC	CC	
Patient type	**N (%)**	**N (%)**	**N (%)**	**p-value**
Familial	96 (48)	91 (45)	15 (7)	0.778
MPM	34 (49)	33 (47)	3 (4)	
Primary Melanomas				
1	70 (45)	73 (47)	12 (8)	
2	45 (50)	40 (44)	5 (6)	0.857
3 or more	15 (55)	11 (41)	1 (4)	
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**p-value**
Age at diagnosis	44 (16)	45 (16)	50 (18)	0.445

Familial: melanoma patients with family history of melanoma in first or second degree relatives, independent of the total number of primary melanomas

MPM: sporadic primary melanoma patient

ARTICLE 4

Genome-wide linkage analysis in Spanish melanoma-prone families identifies a new familial melanoma susceptibility locus at 11q.

Potrony M, Puig-Butille JA, Farnham JM, Giménez-Xavier P, Badenas C, Tell-Martí G, Aguilera P, Carrera C, Malvehy J, Teerlink CC, Puig S.

Eur J Hum Genet. 2018 (published online 30-April-2018)..

Aim

To identify new familial melanoma susceptibility loci.

Patients

29 melanoma patients and 39 non-affected individuals, belonging to 11 melanoma-prone families visited at the Melanoma Unit – Hospital Clínic of Barcelona:

- 10 *CDKN2A*-negative families
- one family with *CDKN2A*-positive and two *CDKN2A*-negative cases

Methods

Subjects were genotyped on either the HumanOmni2.5 (Illumina). We reduced the > 2.3 million SNPs to a non-linkage disequilibrium set, which resulted in 24,225 SNPs for analysis.

Mcsim software was used to perform parametric linkage analysis. The program calculates robust multipoint LOD scores (referred to as TLODs) and evidence from multiple pedigrees was assessed with the heterogeneity-TLOD statistic (het-TLOD).

For locus significance assessment, we used: LOD > 0.588 for nominal evidence, > 1.9 for suggestive evidence, and > 3.3 for significant evidence.

Variables included in analyses

Individual status (melanoma, non-melanoma).

Main results

In the analysis using evidence from multiple families, we identified a region with a maximum het-TLOD of 3.449 (rs12285365:A>G) and spanned the 11q14.1-q14.3 locus (when using one het-TLOD score support interval). The subregions with the strongest evidence contained four protein-coding genes: *DLG2*, *PRSS23*, *FZD4*, and *TMEM135*.

We also detected several regions with suggestive linkage evidence (TLOD > 1.9) (1q, 6p, 7p, 11q, 12p, 13q) including the region previously detected in melanoma-prone families from Sweden at 3q29

We detected three regions with suggestive evidence for linkage in family #1. The first region was in 1q31.1-q32.1 with a maximum TLOD of 2.447 (rs2246083:G>A and rs11590469:C>T), the second region was in 6p24.3-p22.3 with a maximum TLOD of 2.409 (rs4712415:T>C), and the third region was in 11q13.3-q21 with a maximum TLOD of 2.654 spanning >100 markers.

European Journal of Human Genetics
https://doi.org/10.1038/s41431-018-0149-8

ARTICLE

Genome-wide linkage analysis in Spanish melanoma-prone families identifies a new familial melanoma susceptibility locus at 11q

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Received: 10 August 2017 / Revised: 23 February 2018 / Accepted: 27 March 2018

Abstract

The main genetic factors for familial melanoma remain unknown in >75% of families. *CDKN2A* is mutated in around 20% of melanoma-prone families. Other high-risk melanoma susceptibility genes explain <3% of families studied to date. We performed the first genome-wide linkage analysis in *CDKN2A*-negative Spanish melanoma-prone families to identify novel melanoma susceptibility loci. We included 68 individuals from 2, 3, and 6 families with 2, 3, and at least 4 melanoma cases. We detected a locus with significant linkage evidence at 11q14.1-q14.3, with a maximum het-TLOD of 3.449 (rs12285365: A>G), using evidence from multiple pedigrees. The genes contained by the subregion with the strongest linkage evidence were: *DLG2, PRSS23, FZD4*, and *TMEM135*. We also detected several regions with suggestive linkage evidence (TLOD >1.9) (1q, 6p, 7p, 11q, 12p, 13q) including the region previously detected in melanoma-prone families from Sweden at 3q29. The family-specific analysis revealed three loci with suggestive linkage evidence for family #1: 1q31.1-q32.1 (max. TLOD 2.447), 6p24.3-p22.3 (max. TLOD 2.409), and 11q13.3-q21 (max. TLOD 2.654). Future next-generation sequencing studies of these regions may allow the identification of new melanoma susceptibility genetic factors.

Introduction

Melanoma etiology is complex and involves environmental, phenotypic, and genetic factors. Approximately 10% of melanoma cases occur in a familial context. To date, *CDKN2A* (NG_007485.1, NM_000077.4 (p16INK4A) and NM_058195.3 (p14ARF), LRG_11) is the main high-risk susceptibility gene and germline pathogenic variants are detected in around 20% of melanoma-prone families worldwide [1]. The prevalence of *CDKN2A* pathogenic variants varies across populations (5–72%) [2]. In the Mediterranean population, due to the low incidence of the disease, melanoma-prone families are considered as those with at least two melanoma patients in first- or second-degree relatives [3, 4]. Overall, 14% of Spanish melanoma-prone families carry *CDKN2A* pathogenic variants, with prevalence increasing with the number of cases in the family: 11% in families with 2 cases, 23% in families with 3 cases, and 36–43% in families with at least 4 melanoma cases [5, 6].

Beyond *CDKN2A*, other high-risk melanoma genes have been identified, but they account for <3% of the families studied worldwide [1]. Thus, the genetic factors underlying melanoma susceptibility remain unknown in a substantial number of high-risk melanoma families [1].

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These authors jointly supervised this work: Craig C. Teerlink, Susana Puig.

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41431-018-0149-8) contains supplementary material, which is available to authorized users.

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Published online: 30 April 2018

Previous genome-wide linkage analyses, either using microsatellite marker sets or high-density single-nucleotide polymorphism (SNP) arrays, have been conducted in *CDKN2A* wild-type melanoma-prone families, mostly from pedigrees of Northern European ancestry [7–9]. Altogether, these studies suggest 1p22, 9q21, and 17p12-p11 as melanoma susceptibility loci. Notably, the regions detected in these studies were restricted to each geographic population without overlap between studies, and results typically achieved suggestive evidence for linkage. To date, only one study has been conducted in Mediterranean melanoma pedigrees from Italy [10], which failed to detect results with suggestive or significant linkage evidence.

With the goal of identifying new familial melanoma susceptibility loci, we report the first genome-wide linkage analyses conducted in Spanish melanoma-prone families. This is the first study carried out in Mediterranean melanoma pedigrees that has been able to detect genomic regions reaching significant genome-wide linkage evidence.

Subjects and methods

Samples and pedigrees

The study included 29 melanoma cases and 39 non-affected individuals belonging to 11 Spanish melanoma-prone families (10 *CDKN2A*-negative families and one family with *CDKN2A*-positive and two *CDKN2A*-negative cases), with genome-wide genotyping data available from at least two melanoma cases (Figure S1). The family set was enriched with families with a high number of cases for our geographic location: six families with ≥4 melanoma cases, three families with 3 melanoma cases, and two families with 2 melanoma cases. All patients belonged to melanoma-prone families under dermatological follow-up at the Melanoma Unit of Hospital Clinic of Barcelona. For family and individual de-identification, the families included in the study were numbered consecutively from 1 to 11 and sex has been hidden on purpose.

The study was approved by the ethical committee of Hospital Clinic of Barcelona. All patients provided written, informed consent.

Linkage analysis

Subjects were genotyped on either the HumanOmni2.5 (Illumina) array versions v1.0 (81% of subjects) or v1.1 (19% of subjects). The GEO accession number for the genotyping data reported in this paper is GSE109208. Only SNPs common to both versions were included in the study (2,426,511 SNPs). We also excluded SNPs with missing genotypes in >95% of samples (2,332,767 SNPs remaining). Since linkage disequilibrium between markers can artificially inflate evidence for linkage [8], we reduced the set of markers to a non-linkage disequilibrium set by iteratively removing markers with heterozygosity <0.3, $r^2 >$ 0.16 with a previously selected marker and a minimum distance of 0.1 cm between markers, which resulted in 24,225 SNPs for analysis [8].

Mcsim software was used to perform parametric linkage analysis. Mcsim uses Monte Carlo Markov Chain techniques to provide haplotype reconstructions to extract inheritance information in pedigrees [11]. In addition to standard multipoint logarithm of the odds (LOD) scores, the program calculates robust multipoint LOD scores (referred to as TLODs). TLOD score is preferable to standard multipoint because it incorporates the recombination frequency (theta) in the statistic's parameterization, preserving the robustness of the two-point LOD statistic to model misspecification while taking advantage of multipoint information [12]. The TLOD statistic follows the same theoretical distribution as other LOD score statistics (e.g., two-point, multipoint, and heterogeneity-LOD (het-LOD) scores) and can be interpreted with the same conventions. Lander and Kruglyak proposed using LOD >0.588 for nominal evidence, >1.9 for suggestive evidence, and >3.3 for significant evidence [13]. Evidence from multiple pedigrees was assessed with the heterogeneity-TLOD statistic (het-TLOD) [14]. Allele frequencies were estimated internally and general dominant and recessive models were used. We analyzed all pedigrees using an affected-only model that assumed a disease gene frequency of 0.005 for a dominant model and 0.05 for a recessive model. The penetrance estimates for carriers and non-carriers were 0.5 and 0.0005, respectively. The genome version GRCh37/hg19 was used to establish genomic positions. Reference sequence (RefSeq) database at NCBI and GeneCards Human Gene Database (http://www.genecards.org/) were used to obtain information on the genomic features in the regions of interest [15, 16].

Haplotype phasing

Regions of interest linked to multiple families were assessed for the presence of common haplotypes shared between linked families. The software SHAPEIT2 was used for haplotype phasing [17]. All genotyped SNPs in regions of interest were used for phasing.

Results

Genome-wide linkage analysis

The het-TLOD genome-wide analysis, using evidence summed across the pedigrees, revealed a region with

Table 1 Genome-wide suggestive het-TLODs (>1.9) and significant het-TLODs (>3.3)

Cytogenetic band	Genome location (GRCh37/hg19)	Model	Maximum score	SNP ID	Gene
1q31.1-q32.1	chr1: 186,946,386–205,336,875	Dom	2.456	rs7517688	None
3q29	chr3: 194,219,913–197,744,198	Rec	2.099	rs11185544	None
6p24.3-p23	chr6: 8,193,128–15,089,151	Dom	2.024	rs6925772	*HIVEP1*
7q21.11-q21.2	chr7: 85,216,272–91,167,397	Dom	2.546	rs10268943	None
11q14.1-q14.3	**chr11: 82,498,536–92,106,781**	**Dom**	**3.449**	**rs12285365**	***PRSS23***
11q22.1	chr11: 97,804,083–100,498,349	Dom	2.462	rs17577073	*CNTN5*
12p13.1	chr12: 13,066,220–13,617,099	Rec	1.950	rs12815655	None
13q12.3-q14.11	chr13: 30,484,862–43,478,867	Rec	2.365	rs2312972	None

The threshold used to define the boundary region was 1 het-TLOD difference from the maximum regional score. The significant het-TLODs (>3.3) are highlighted in bold

Dom dominant, *Rec* recessive

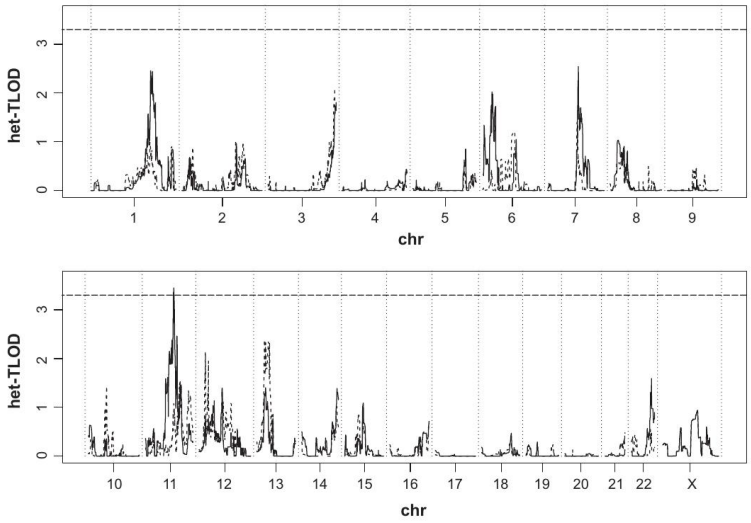

Fig. 1 Genome-wide het-TLOD scores. Genome-wide het-TLOD scores in dominant (continuous line) and recessive (dashed line) models are plotted. Significant linkage evidence threshold (het-TLOD >3.3) is denoted by the horizontal dashed line

significant linkage (het-TLOD >3.3) on chromosome 11 (Table 1 and Fig. 1). This region had a maximum het-TLOD of 3.449 (rs12285365:A>G) and spanned the 11q14.1-q14.3 locus (when using one het-TLOD score support interval). The region contains 52 genomic features of which 38 are protein-coding genes. The strongest linkage evidence at this locus (all markers with het-TLOD >3.3) was detected in two regions: between rs1940085:G>A and rs7108021:T>G (chr11: 84.3–84.6 Mb) and between rs12285365:A>G and rs607530:T>C (chr11: 86.6–87.6 Mb). These regions contain four protein-coding genes: *DLG2* (NG_021375.1, NM_001142699.1), *PRSS23* (NM_007173.5), *FZD4* (NG_011752.1, NM_012193.3), and *TMEM135* (NM_022918.3). We phased haplotypes in the linked pedigrees using all available markers to determine whether the same haplotypes appeared in multiple linked families, but failed to identify any such haplotype. Other regions showed suggestive linkage evidence (TLOD >1.9) at chromosome 1q, 6p, 7p, and 11q under a dominant model, and at chromosome 3q, 12p, and 13q under a recessive model (Table 1). SNPs description based on a genomic reference sequence is shown in Table S1.

Family-specific genome-wide linkage analysis

Data from previous studies suggest that certain high-risk melanoma factors may be restricted to a limited number of pedigrees such as germinal variants in *TERT* (NG_009265.1, NM_198253.2, LRG_343) [18, 19]. Thus, we conducted a separate genome-wide analysis for each family. We detected three regions with suggestive evidence for linkage (TLOD >1.9) in family #1 under a dominant

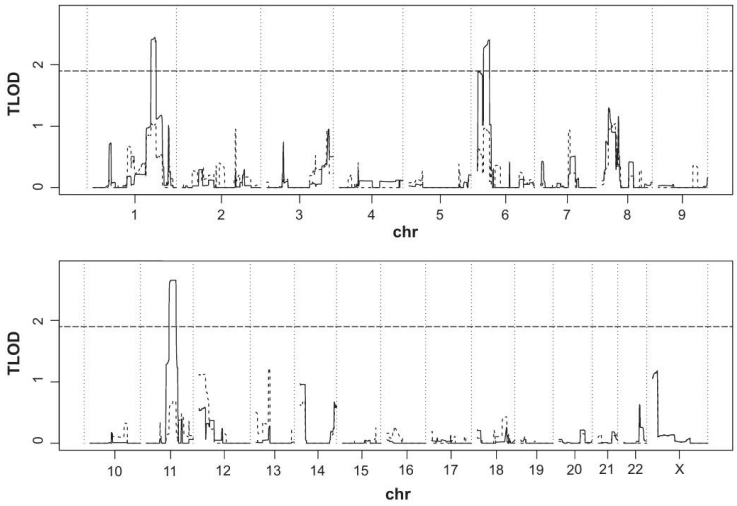

Fig. 2 Genome-wide TLOD scores for family #1. Genome-wide TLOD scores for dominant (continuous plot line) and recessive (dashed plot line) models are plotted. The suggestive linkage evidence threshold (TLOD >1.9) is denoted by the horizontal dashed line

model (Fig. 2). This is a family with six *CDKN2A*-positive melanoma cases, two *CDKN2A*-negative melanoma cases, and other cancers in blood relatives (liver, lung, cervix, endometrial, and breast cancer cases). The *CDKN2A*-negative cases developed melanoma at a young age (32, 33 years old (y.o)) similar to the *CDKN2A*-positive cases (27, 34, 37, 37 y.o). Since, *CDKN2A*-negative cases did not carry medium melanoma risk variants such as *MC1R* (NG_012026.1, NM_002386.3) red-hair color variants or *MITF* (NG_011631.1, NM_000248.3, LRG_776) variant c.952G>A (p.(Glu318Lys)), we hypothesized that the melanoma risk observed in *CDKN2A*-negative cases may result from other melanoma susceptibility variants. We genotyped the *CDKN2A*-negative melanoma cases along with two *CDKN2A*-positive melanoma cases. The analyses identified three regions that segregate with all melanoma cases independently of *CDKN2A* status. The first region was in 1q31.1-q32.1 (chr1: 187.5–205.3 Mb) with a maximum TLOD of 2.447 at markers rs2246083:G>A and rs11590469:C>T (Figure S2). This region spans 17.8 Mb and contains 133 genomic features, of which 103 are protein-coding genes. The second region was in 6p24.3-p22.3 (chr6: 8.2–19.5 Mb) with a maximum TLOD of 2.409 at marker rs4712415:T>C (Figure S3). The region spans 11.3 Mb and contains 63 genomic features, of which 44 are coding protein genes. The third region was in 11q13.3-q21 (chr11: 68.7–95.5 Mb) with a maximum TLOD of 2.654 spanning >100 markers (Figure S4). This region spans 26.8 Mb and contains 239 genetic features, of which 171 are protein-coding genes.

Discussion

In Spain, the genetic background in melanoma-prone families remains unknown in >80% of families [5, 6]. Linkage analysis is likely to detect regions containing high-risk variants or genetic features segregating with the disease. Here, we report the results of a genome-wide linkage screen performed on 11 melanoma-prone families in which we detected significant linkage to the 11q14.1-q14.3 locus for melanoma susceptibility. Although the number of families included in the study is lower than previous studies, the subset of families was enriched by inclusion of highly informative families since 54.4% families had ≥4 melanoma cases.

A previous genome wide association study (GWAS) study performed in melanoma patients reported a melanoma locus at the 11q14.3 region. The study detected the strongest evidence of association near rs1393350:G>A encompassing *TYR* (NG_008748.1, NM_000372.4) gene, which plays a key role in human pigmentation and is a low-risk melanoma gene [20]. In the present study, the two subregions with strongest linkage evidence within 11q14.1-q14.3 do not include the *TYR* gene, suggesting that this genomic region is associated with melanoma susceptibility due to genetic factors other than pigment related alleles in the *TYR* gene. The *DLG2*, *PRSS23*, *FZD4*, and *TMEM135* genes are located in the regions with the strongest linkage evidence. The biological information about this set of genes is limited, but they are all plausible candidates for cancer susceptibility [21, 22]. However, further sequencing data and molecular studies are necessary to elucidate the possible role of these genes in melanoma susceptibility.

Moreover, we have detected seven additional loci (1q31.1-q32.1, 3q29, 6p24.3-p23, 7q21.11-q21.2, 11q22.1, 12p13.1, 13q12.3-q14.11) with suggestive linkage evidence in the studied families. Notably, the 3q28-q29 locus has been previously detected with suggestive evidence of melanoma linkage in *CDKN2A* wild-type Swedish families [9].

A subsequent analysis of those families reported a narrower region spanning 3.5 Mb (chr3: 192.1–195.6 Mb) [23], overlapping the linked region detected in Spanish families. The rest of the suggestive regions detected have not been previously reported. The finding of a common region in Spanish and Swedish melanoma-prone families, strongly suggests that this region may contain genetic factors associated with melanoma susceptibility. The overlap region from both populations contains 20 genetic features, of which 10 are protein-coding genes (Table S2) including plausible candidates involved in proliferation and apoptosis, lipid transport, serin/threonin phosphatase PP1 inhibition, or Notch activation [24–27].

Melanoma is one of the tumors with highest heritability [28]. In families with melanoma aggregation, melanoma susceptibility follows an autosomal dominant inheritance pattern with incomplete penetrance. Multiple genes can play a role in melanoma susceptibility in a family, by combination of high-risk gene/s and presence of medium-/low-risk variants modulating expressivity of the high-risk gene/s. High-risk variants or genetic features segregate with the disease in most affected cases in the family and may be detected by linkage analysis studies. We expect to identify one or very few high-risk variants in an individual. However, in >70% of families worldwide, these have still not been identified. Thus, studies such as the present one are needed to provide clues to new genomic regions to focus on in order to identify new high-risk variants that may explain part of the missing heritability of melanoma susceptibility. The combination of medium-/low-risk variants modulates the penetrance and expressivity of high-risk genes, but may vary within the family and may be inherited from different ancestors. Multiple medium-/low-risk variants have been described to date [1]. However, their specific role in the modulation of the expressivity of pathogenic variants in high-risk genes has only been well established for the highly polymorphic pigmentation gene *MC1R*. *CDKN2A* variant carriers with melanoma-associated variants in *MC1R* have an increased risk of developing melanoma than *CDKN2A* variant carriers with wild-type *MC1R* [29]. Although co-existence of a *CDKN2A* pathogenic variant with the rare *MITF* c.952G>A (p.(Glu318Lys)) variant has also been described [30], the implication of *MITF* in the modulation of melanoma penetrance in *CDKN2A* variant carriers is still unknown.

In our study, we included a *CDKN2A*-positive family in which two melanoma cases did not carry known high-risk nor medium-risk melanoma susceptibility variants. We detected three loci with suggestive linkage evidence indicating that, in addition to the *CDKN2A* pathogenic variant, other genetic factors underlie the increased melanoma risk observed in the members of this family. Knowing the gene, or combination of genes, involved in melanoma susceptibility is crucial for identification and better management of at-risk individuals. Furthermore, it allows the refinement of genetic counseling in melanoma, as specific measures can be included when genetic testing detects germline variants in known susceptibility genes [5, 30–32].

In conclusion, using linkage evidence from multiple pedigrees, we have identified a familial melanoma susceptibility locus at 11q14.1-q14.3, in Spanish melanoma-prone families. We have also detected suggestive evidence of linkage at 3q29, previously described in Swedish families. Future next-generation sequencing studies or candidate gene targeted sequencing from these regions may allow the identification of new genetic factors implicated in melanoma susceptibility.

Acknowledgements Thanks to our patients and their families who are the main reason for our studies, and to nurses from the Melanoma Unit of Hospital Clínic of Barcelona, D. Gabriel, P. Iglesias, and M.E. Moliner for helping to collect patient data.

Funding The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants 09/1393, 12/00840, and 15/00716; CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain, co-financed by European Development Regional Fund "A way to achieve Europe" ERDF; AGAUR 2014_SGR_603 and 2017_SGR_1134 of the Catalan Government, Spain; European Commission under the 6th Framework Programme, contract no. LSHC-CT-2006-018702 (GenoMEL), by the European Commission under the 7th Framework Programme, Diagnoptics, and by the European Union's Horizon 2020 research and innovation programme, IMMUSPHINX; The National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115), a grant from "Fundació La Marató de TV3, 201331-30", Catalonia, Spain, CERCA Programme/Generalitat de Catalunya, and grant GCB15152978SOEN from "Fundación Científica de la Asociación Española Contra el Cáncer", Spain. Part of the work was carried out at the Esther Koplowitz Center, Barcelona. SNP genotyping services were provided by the "Centro Nacional de Genotipado" (CEGEN-ISCIII, Santiago de Compostela, Spain) (www.cegen.org) and by the "Instituto de investigación sanitaria INCLIVA" (Valencia, Spain). M.P. is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain. Data analysis was performed at the Division of Genetic Epidemiology facilities, University of Utah, Utah, US, while M.P. was doing a lab visit funded by the MV15/00016 mobility fellowship (M-AES) from Instituto de Salud Carlos III, Spain.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Electronic supplementary material

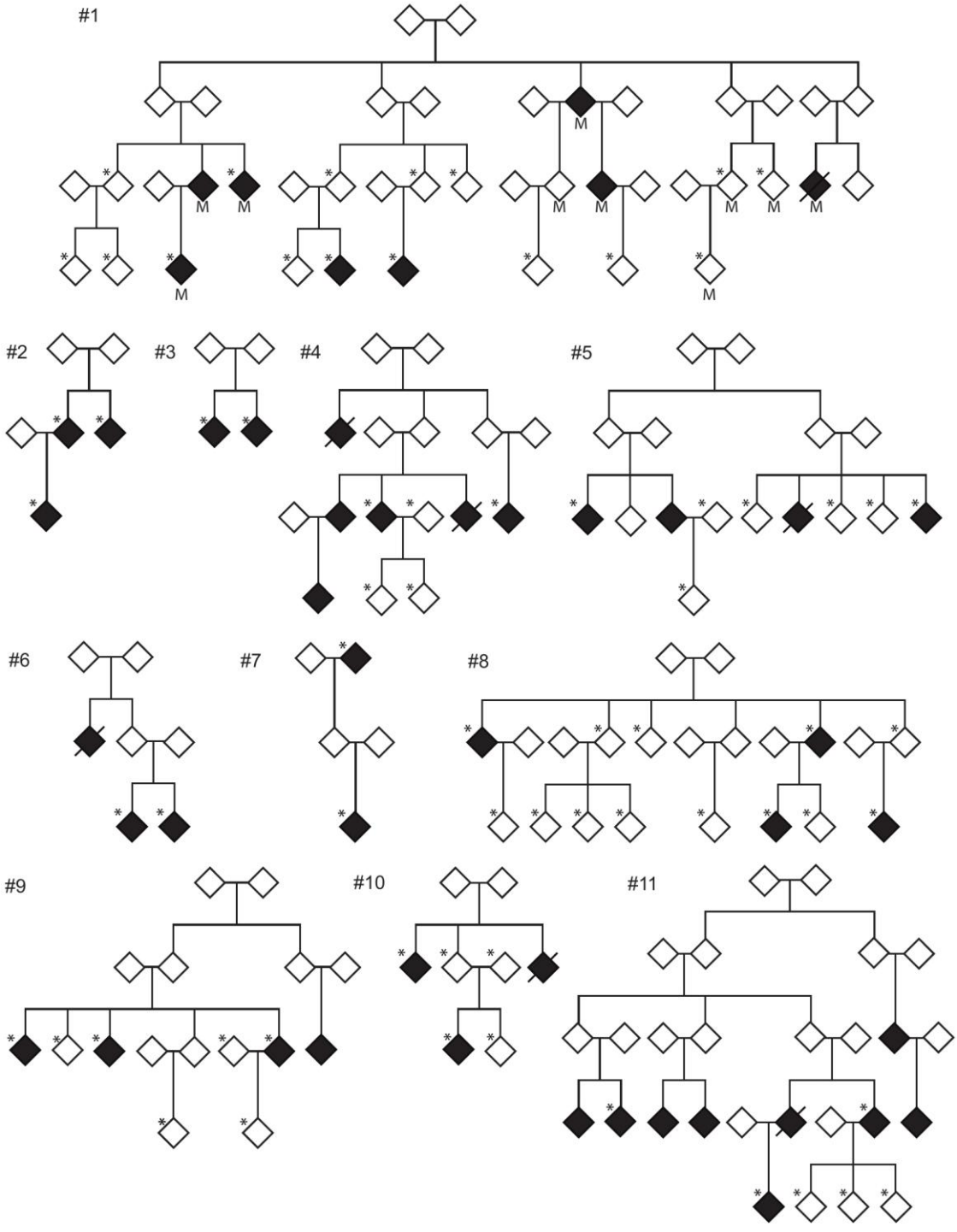
Table S1. SNP description based on a genomic reference sequence based on GRCh37/hg19 human genome version.

dbSNP ID	hg19 genomic sequence
rs2246083:G>A	chr1:g.202781164G>A
rs11590469:C>T	chr1:g.202843127C>T
rs149617956:G>A	chr3:g.70014091G>A
rs4712415:T>C	chr6:g.19525075T>C
rs1940085:G>A	chr11:g.84319959G>A
rs7108021:T>G	chr11:g.84649642T>G
rs12285365:G>A	chr11:g.86572384G>A
rs607530:T>C	chr11:g.87598975T>C
rs1393350:G>A	chr11:g.89011046G>A

Table S2. Protein coding genes in the 3q29 overlapping region from the Swedish and the present studies.

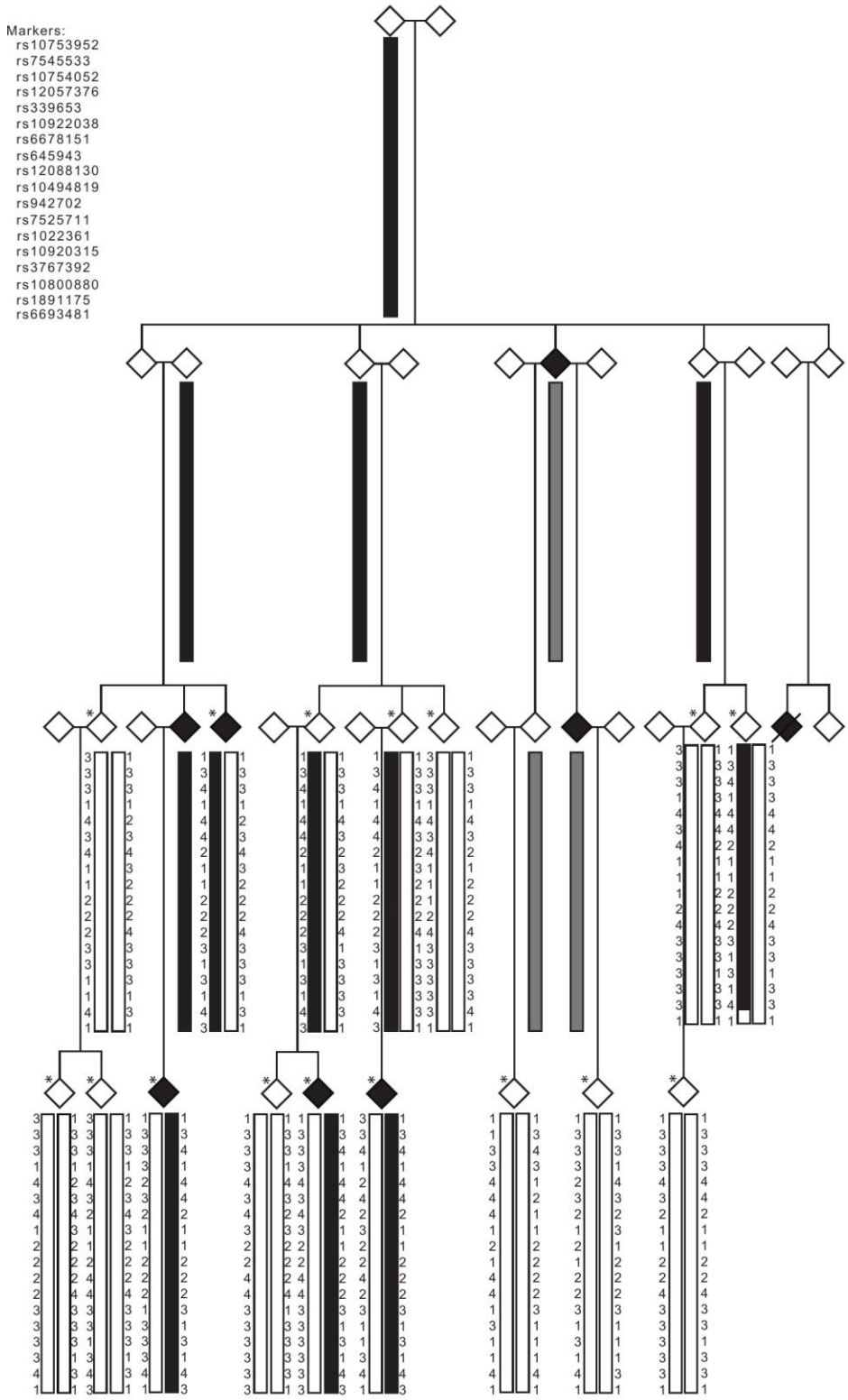
Gene Name	Accession Numbers
ACAP2	NM_012287.5
APOD	NM_001647.3
FAM43A	NM_153690.4
LSG1	NM_018385.2
MUC20	NG_033877.1, NM_001282506.1
MUC4	NG_053117.1, NM_018406.6
PPP1R2	NM_001316325.1
TMEM44	NM_001166305.1
TNK2	NG_029779.1, NM_001010938.1
XXYLT1	NM_152531.4

Figure S1. Pedigrees from families included in the genome-wide linkage analysis.



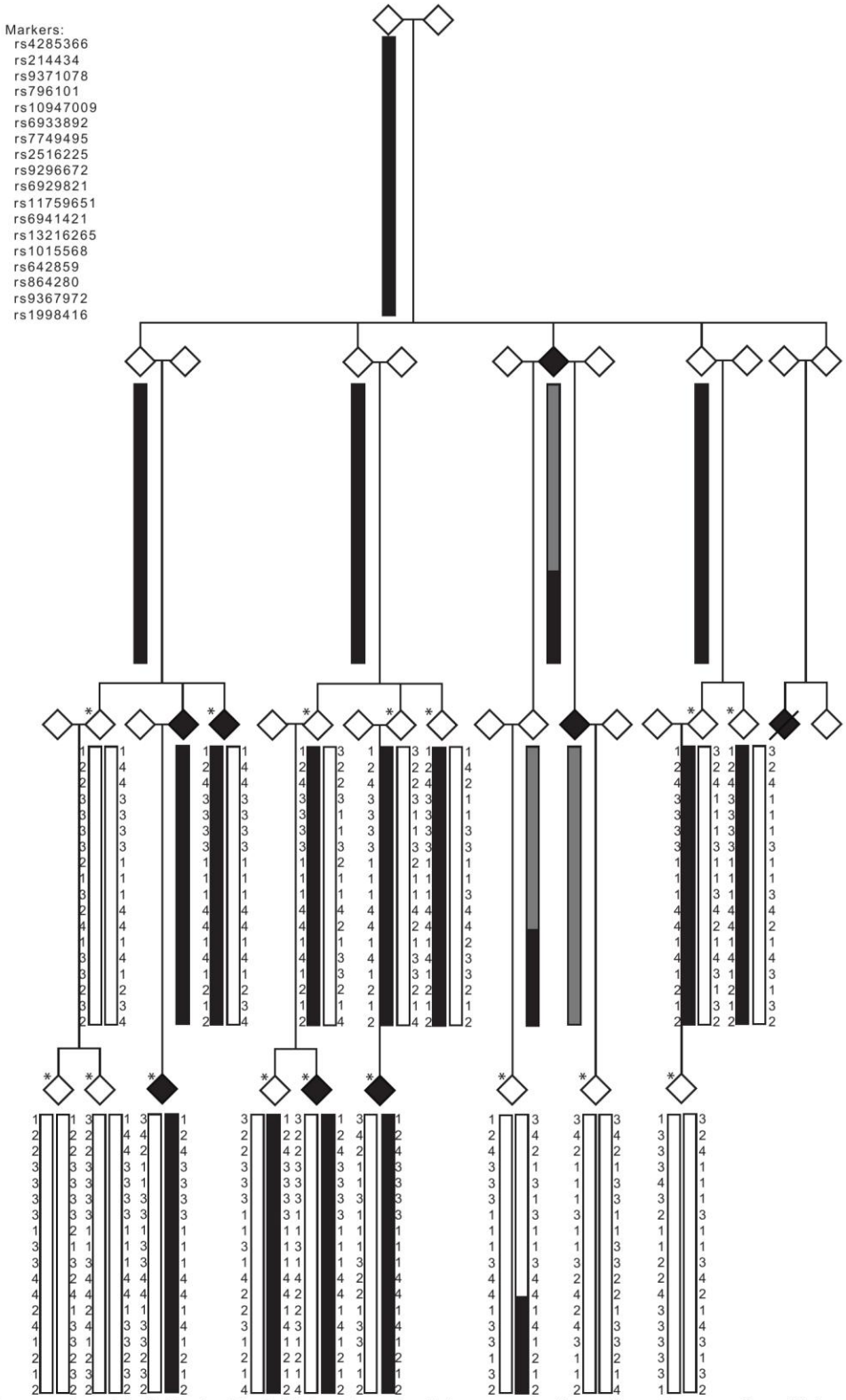
Black symbols indicate melanoma cases. * indicates genotyped individuals. Some individuals in family #1 carried a CDKN2A mutation (M). All other genotyped individuals are CDKN2A wild-type. Pedigree structure was simplified in some families showing all the known affected in each pedigree, all unaffected genotyped, and excluding other unaffected individuals. Sex was hidden on purpose to prevent identification.

Figure S2. Locus 1q31.1-q32.1 haplotype segregation in family #1.



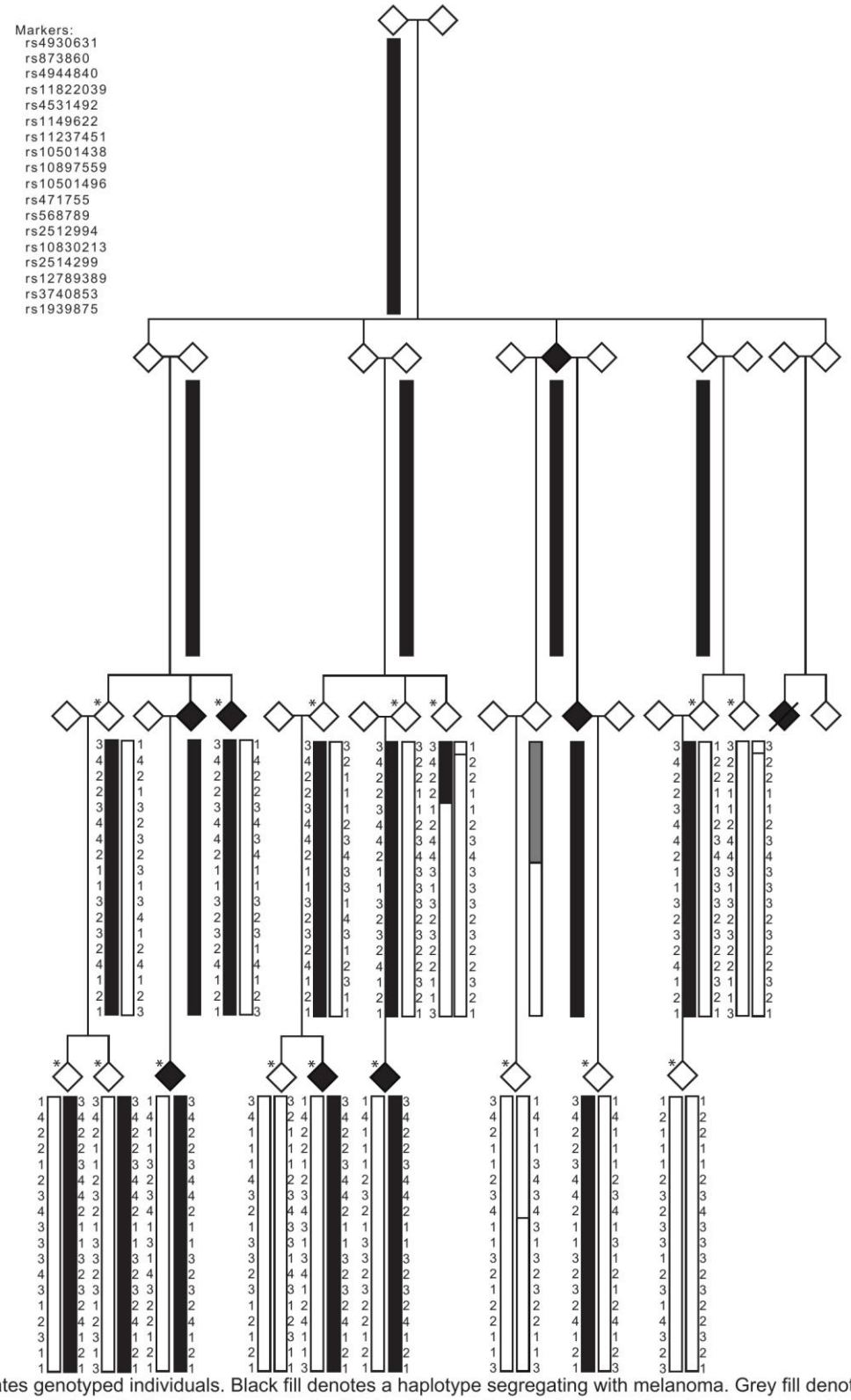
* indicates genotyped individuals. Black fill denotes a haplotype segregating with melanoma. Grey fill denotes unknown haplotype sharing (non-genotyped patients with no evidence of sharing given the descendants' genotypes). White fill denotes other haplotypes. Every fifth marker in the region was used for plotting in order to condense the plot space.

Figure S3. Locus 6p24.3-p22.3 haplotype segregation in family #1.



* indicates genotyped individuals. Black fill denotes a haplotype segregating with melanoma. Grey fill denotes unknown haplotype sharing (non-genotyped patients with no evidence of sharing given the descendants' genotypes). White fill denotes other haplotypes. Every eighth marker in the region was used for plotting in order to condense the plot space.

Figure S4. Locus 11q13.3-q21 haplotype segregation in family #1.



* indicates genotyped individuals. Black fill denotes a haplotype segregating with melanoma. Grey fill denotes unknown haplotype sharing (non-genotyped patients with no evidence of sharing given the descendants' genotypes). White fill denotes other haplotypes. Every tenth marker in the region was used for plotting in order to condense the plot space.

ARTICLE 5

IRF4 rs12203592 functional variant and melanoma survival.

Potrony M, Rebollo-Morell A, Giménez-Xavier P, Zimmer L, Puig-Butille JA, Tell-Marti G, Sucker A, Badenas C, Carrera C, Malvehy J, Schadendorf D, Puig S.

Int J Cancer. 2017;140(8):1845-1849.

Aim

To explore the role of *IRF4* SNP rs12203592 in the modulation of the clinical outcome in two independent melanoma patient sets.

Patients

493 melanoma patients from the Melanoma Unit of the Hospital Clinic of Barcelona, Spain (January 1994 – January 2013)

- median time of follow-up: 42 months

432 melanoma patients from University Hospital Essen, Germany (1982 – 2009)

- median time of follow-up: 52 months

Methods

TaqMan Genotyping Assay was used to genotype *IRF4* SNP rs12203592.

Statistical association and survival analyses.

Variables included in analyses

IRF4 rs12203592 genotype

Age of onset, Gender

Primary melanoma features (Breslow thickness, anatomic site)

Staging information (SLN status, presence of cutaneous or nodal metastasis at diagnosis)

Time of follow-up and status at last follow-up (alive, dead)

Main results

IRF4 rs12203592 T allele:

- increases the risk of dying from melanoma in both sets (Barcelona: OR = 6.53, 95% CI 1.38–30.87, Adj *P* = 0.032; Essen: OR = 1.68, 95% CI 1.04–2.72, Adj *P* = 0.035).
- increases the risk of developing melanoma in head and neck (OR = 1.79, 95% CI 1.07–2.98, Adj *P* = 0.032), while it protected from developing melanoma in the trunk (OR = 0.59, 95% CI 0.41–0.85, Adj *P* = 0.004).

Patients homozygous for the T allele had a worse prognosis when diagnosed at early stages, whereas this effect was lost in patients diagnosed at stage III.

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International Journal of Cancer

Short Report

IRF4 rs12203592 functional variant and melanoma survival

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Inherited genetic factors may modulate clinical outcome in melanoma. Some low-to-medium risk genes in melanoma susceptibility play a role in melanoma outcome. Our aim was to assess the role of the functional *IRF4* SNP rs12203592 in melanoma prognosis in two independent sets (Barcelona, $N = 493$ and Essen, $N = 438$). Genotype association analyses showed that the *IRF4* rs12203592 T allele increased the risk of dying from melanoma in both sets (Barcelona: odds ratio [OR] = 6.53, 95% CI 1.38–30.87, Adj $p = 0.032$; Essen: OR = 1.68, 95% CI 1.04–2.72, Adj $p = 0.035$). Survival analyses only showed significance for the Barcelona set (hazard ratio = 4.58, 95% CI 1.11–18.92, Adj $p = 0.036$). This SNP was also associated with tumour localization, increasing the risk of developing melanoma in head or neck (OR = 1.79, 95% CI 1.07–2.98, Adj $p = 0.032$) and protecting from developing melanoma in the trunk (OR = 0.59, 95% CI 0.41–0.85, Adj $p = 0.004$). These findings suggest for the first time that *IRF4* rs12203592 plays a role in the modulation of melanoma outcome and confirms its contribution to the localization of the primary tumour.

Cancer Epidemiology

Melanoma is the most aggressive of the common skin cancers. In most cases, melanomas diagnosed at early stages can be cured by proper surgical excision, but if diagnosis is delayed, the metastatic rate increases 10% per millimeter thickness.[1] To date, histological features of the primary melanoma such as Breslow tumour thickness, mitotic rate and ulceration – together with the sentinel lymph node (SLN) status – are important hallmarks of early melanoma prognosis and staging.[1,2] However, besides treatment, inherited genetic factors might modulate clinical outcome and possibly explain widely varying survival rates.[3]

Previous studies have identified several genes and single nucleotide polymorphisms (SNPs) that modulate melanoma outcome. Interestingly, some of them are well-known low-to-medium risk susceptibility genes for melanoma.[4] This is the case of Melanocortin 1 Receptor (*MC1R*), the master regulator gene of human pigmentation. *MC1R* is highly polymorphic and some of its functional variants are associated with increased melanoma risk. Two independent studies showed that the presence of variants in *MC1R* improve melanoma-specific survival (hazard ratio [HR] = 0.64, 95% CI 0.46–0.89, $p = 0.01$ and HR = 0.60, 95% CI 1.01–1.39, $p = 0.01$, respectively).[5,6] Loss of function variants in *MC1R* upregulate oxidative stress-related pathways[7] and DNA damage,[8] favouring the apoptosis of damaged cells, which could explain in part this effect on prognosis. The poly (ADP-ribose) polymerase 1 (*PARP1*) SNP rs2249844, which is in linkage with an intronic SNP associated with a reduced risk of melanoma development, is also implicated in melanoma survival (HR=

Key words: melanoma, IRF4, survival, prognosis, genetics
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Spanish Fondo de Investigaciones Sanitarias; **Grant numbers:** 12/00840 and 15/00716; **Grant sponsor:** CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain; **Grant sponsor:** European Development Regional Fund "A way to achieve Europe" ERDF; **Grant sponsor:** AGAUR 2014_SGR_603 of the Catalan Government, Spain; **Grant sponsor:** European Commission under the 6th Framework Programme; **Grant number:** LSHC-CT-2006–018702 (GenoMEL); **Grant sponsor:** European Commission under the 7th Framework Programme, Diagnoptics; **Grant sponsor:** The National Cancer Institute (NCI) of the US National Institute of Health (NIH); **Grant number:** CA83115; **Grant sponsor:** Fundació La Marató de TV3, Catalonia, Spain; **Grant number:** 201331–30; **Grant sponsor:** CERCA Programme/Generalitat de Catalunya; **Grant sponsor:** Fundación Científica de la Asociación Española Contra el Cáncer, Spain
DOI: 10.1002/ijc.30605
History: Received 28 Sep 2016; Accepted 3 Jan 2017; Online 19 Jan 2017
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Int. J. Cancer: **140**, 1845–1849 (2017)

What's new?

Certain genetic factors are thought to increase the risk of developing melanoma. Some of these factors also appear to influence clinical outcome, in addition to initial melanoma risk. For example, a high nevi (mole) count has been associated with better survival. In this study, the authors found that a single nucleotide polymorphism the gene *IRF4* influences nevi count, tumour localization and risk of dying from melanoma. These results suggest that *IRF4* may thus play a role in the association between nevi count and melanoma prognosis.

1.20, 95% CI 1.01–1.39, $p = 0.03$).[9,10] Finally, several SNPs in *VDR* (Vitamin D Receptor) playing a role in melanoma susceptibility[11] have been associated with melanoma survival.[12]

Interferon regulatory factor 4 (*IRF4*) SNP rs12203592 is associated with melanoma susceptibility.[13–15] IRF4 is a transcription factor belonging to the interferon regulatory family, also expressed in melanocytic lesions.[16] Furthermore, the T allele of rs12203592 represses its promoter activity in melanocytes.[17] Our aim was to explore the role of *IRF4* SNP rs12203592 in the modulation of the clinical outcome in two independent melanoma patient sets.

Material and Methods

Design and samples

This retrospective study comprised two independent hospital-based series of melanoma patients. The recruitment, and therefore blood sampling, took place, where possible, 3–6 months after diagnosis. Inclusion criteria were confirmed alive/death status at last follow-up (melanoma-specific survival), at least one update in follow-up (months) since the date of diagnosis, gender (male or female), age at diagnosis and Breslow thickness (mm). Exclusion criteria were lack of germinal DNA sample and lack of signed informed consent.

The first set consisted of a hospital-based series of 493 melanoma patients from the Melanoma Unit of the Hospital Clinic of Barcelona, Spain. Patients were diagnosed with melanoma between 1994 and January 2013 (median time of follow-up: 42 months). The disease status was established through the annual review and review of medical notes, from electronic records of the patients visited every 3–4 months for the first 2 years, then every 6 months until 5 years and annually until 10 years. Primary tumour localization was available for all patients. The primary tumour localization was classified as head and neck, upper extremities, lower extremities, trunk, acral or mucosal.

The second set comprised a hospital-based series of 432 melanoma patients from University Hospital Essen, Germany. Patients were diagnosed with melanoma between 1982 and 2009 (median time of follow-up was 52 months). The disease status was established in the same way as for the Barcelona set. It also included an update of loss of follow-up by phone calls. Specific primary tumour localization was not available.

The patient's stage at diagnosis according to AJCC[1] in the Barcelona set was 45% stage I (23 IA, 186 IB, 13 I unknown ulceration status), 33% stage II (51 IIA, 52 IIB, 17 IIC, 44 II unknown ulceration status) and 22% stage III (number of positive SLN or presence of micro/macro metastasis was not recorded in our database, thus patients could not be subclassified into IIIA, IIIB or IIIC). The patient's stage at diagnosis according to AJCC in the Essen set was 31% stage I (133 IB), 49% stage II (83 IIA, 68 IIB, 27 IIC, 36 II unknown ulceration status) and 20% stage III (26 IIIA, 23 IIIB, 8 IIIC and 28 III with unknown number of positive SLN or presence of micro/macro metastasis).

The study was approved by the ethical committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

IRF4 genotyping

Genomic DNA was obtained from peripheral blood lymphocytes. TaqMan Genotyping Assay was used to genotype *IRF4* SNP rs12203592 (assay C__31918199_10, Life Technologies) in all patients, following the manufacturers recommendations. The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used. The SNP was in Hardy–Weinberg equilibrium in both sets (Barcelona: $p = 0.880$; Essen: $p = 1.000$).

Statistical analyses

Genotype association analyses were performed using multiple logistic regression models (co-dominant, dominant, recessive, over-dominant and log additive). The selection of the most suitable model of inheritance was performed based on both the Akaike information criterion and the Bayesian information criterion. Once the model was determined, differences in genotypic frequencies were calculated using a two-sided Pearson chi-squared test. These analyses were performed using the bioinformatics tool SNPStats (http://bioinfo.iconcologia.net/SNPstats).[18]

Two-sided Pearson chi-squared test was used for general descriptive analyses for categorical variables. *t*-test was used for general descriptive analyses for continuous variables. Breslow thickness variable did not follow the normal distribution and was transformed using the logarithm function. Melanoma-specific survival according to *IRF4* genotype was assessed using Kaplan–Meier curves and Cox regression multivariate analyses. SPSS 17.0 was used to perform descriptive statistical analyses and survival analyses.

Gender, age at diagnosis, Breslow logarithm and stage at diagnosis were included as covariates in the analyses. Primary tumour localization analyses according to *IRF4* genotype

Table 1. Genetic association of *IRF4* SNP rs12203592 with melanoma-specific survival

	MAF (T allele) *N* (%)		Genotype frequency *N* (%)								
			Alive			Dead					
	Alive	Dead	CC	CT	TT	CC	CT	TT	OR	95% CI	Adj *p*
Barcelona (*N* = 493)	145 (16%)	13 (20%)	325 (71%)	125 (27%)	10 (2%)	23 (70%)	7 (21%)	3 (9%)	**6.53**	**1.38–30.87**	**0.032**
Essen (*N* = 432)	55 (10%)	46 (14%)	215 (81%)	47 (18%)	4 (2%)	122 (73%)	42 (25%)	2 (1%)	**1.68**	**1.04–2.72**	**0.035**

Adj *p*: *p* values were adjusted by clinical covariates (age at diagnosis, gender, Breslow logarithm and stage at diagnosis). In the Barcelona set, *p* values were also adjusted by primary tumour anatomic location. The ORs, CIs and *p* values are based on genotype frequencies. The genetic models used to obtain the OR, 95% CI and Adj *p* values were the recessive in Barcelona and the dominant in Essen. Statistically significant results are highlighted in bold.
Abbreviation: MAF, minor allele frequency.

were corrected by age and gender. The tests were considered significant if *p* or Adj *p* as applicable was <0.05.

Results

We genotyped the SNP *IRF4* rs12203592 in two independent melanoma patient sets (Barcelona, $N = 493$; Essen, $N = 432$). First, we explored its role in melanoma-specific survival by genotype association analyses. The *IRF4* rs12203592 T allele increased the risk of dying from melanoma in both sets (Barcelona: OR = 6.53, 95% CI 1.38–30.87, Adj $p = 0.032$; Essen: OR = 1.68, 95% CI 1.04–2.72, Adj $p = 0.035$) (Table 1). There were statistically significant differences between the genotype frequency in Barcelona and Essen ($p = 0.029$). The assessment of the melanoma-free survival using Kaplan–Meier and Cox regression analyses also showed that the *IRF4* rs12203592 T allele in homozygosis worsens melanoma-specific survival in the Barcelona set (Fig. 1). As therapeutic strategies for melanoma patients changed dramatically after 2011, we repeated the analyses excluding patients diagnosed in 2011, 2012 and 2013, obtaining similar results (Supporting Information). No statistical significance was reached in the Essen set (Fig. 1). When grouping sets, we saw that patients homozygous for the T allele had a worse prognosis when diagnosed at early stages, whereas this effect was lost in patients diagnosed as stage III (Supporting Information).

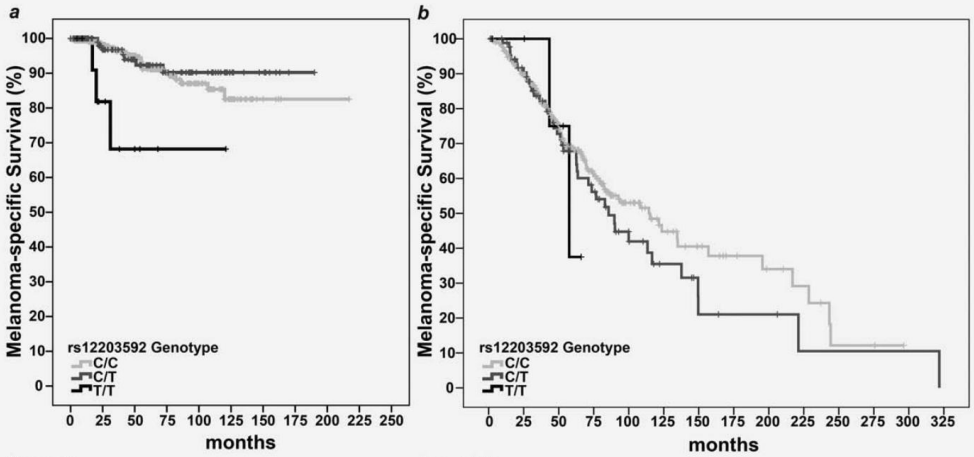
Furthermore, the association analyses between *IRF4* rs12203592 and the location of the primary tumour in the Barcelona set indicated that the *IRF4* rs12203592 T allele increased the risk of developing melanoma in head and neck (OR = 1.79, 95% CI 1.07–2.98, Adj $p = 0.032$), while it protected from developing melanoma in the trunk (OR = 0.59, 95% CI 0.41–0.85, Adj $p = 0.004$) (Table 2). No association was detected between Breslow thickness or stage at diagnosis and *IRF4* rs12203592 in these sets (Supporting Information).

Discussion

Our study showed, for the first time, that the *IRF4* rs12203592 T allele was associated with worse melanoma outcome. Genotype association analyses with melanoma-specific survival were statistically significant in the two sets studied. However, survival curve analyses were only significant in the Barcelona set. The Essen set included a higher proportion of patients diagnosed at stage III and, in general, had a worse prognosis than in Barcelona (thicker Breslow, higher male proportion and older age at diagnosis). Together with the fact that the T allele was less common in Essen and the number of homozygous TT patients was very low, this could explain why we did not detect differences in survival curves between groups in the Essen set.

The *IRF4* rs12203592 T allele has been previously associated with a protective role in melanoma susceptibility,[15] while the C allele increases the risk of developing melanoma.[13] In adults, the *IRF4* rs12203592 C allele is associated with a high nevi count.[13,14] Ribero *et al.* reported that high nevi count, besides increasing melanoma risk, confers a favourable prognosis in melanoma.[19] Consistent with their results, in our study, the *IRF4* rs12203592 T allele confers a worse melanoma prognosis. Ribero *et al.* suggested that melanoma patients with high nevi count may have improved survival because the genetic determinants for nevi count may be associated with biological differences in the tumour.[19] Our results suggest that *IRF4* may be one of the genes explaining this association.

IRF4 rs12203592 affects a melanocyte-specific enhancer regulator. The T allele impairs the function of this enhancer, leading to a reduction of *IRF4* expression.[17] MITF and TFAP2A bind this element when the C allele is present, activating *IRF4* expression in melanocytes.[17] IRF4 activates the melanogenesis pathway by the regulation of *TYR* expression, together with MITF. This could be one explanation of why these genes are both implicated in nevi count.[13,20,21] It has been suggested that *IRF4* expression enables suppressive regulatory T cells (Treg) to suppress effector T cells.[22] In lymphocytes, contrary to melanocytes, the C allele of *IRF4* rs12203592 inhibits the expression of this gene.[23] Thus, individuals carrying the T allele, have a higher expression of *IRF4* in lymphocytes. Tregs from these individuals may have a higher ability to inhibit the immune response against the tumour, explaining why these individuals have a worse melanoma prognosis. Correlating with this, we have identified that functional variants inherited in a negative T cell



c

Group	Genotype	N	5-year survival rate	HR	95%CI	Adj P
Barcelona	CC	348	0.91	Ref.		
	CT	132	0.92	0.80	0.33-1.96	0.619
	TT	13	0.68	4.58	1.11-18.92	**0.036**
Essen	CC	337	0.69	Ref.		
	CT	89	0.68	1.24	0.87-1.78	0.234
	TT	6	0.38	1.76	0.43-7.25	0.434

Figure 1. Melanoma-specific survival curves according to *IRF4* rs12203592 genotype. (*a*) Kaplan–Meier curves for Barcelona set. Log Rank test $p = 0.005$. (*b*) Kaplan–Meier curves for Essen set. Log Rank test $p = 0.521$. (*c*) Cox regression analyses results. The hazard ratio (HR) and *p* values were adjusted by age at diagnosis, gender, Breslow logarithm, stage at diagnosis and primary tumour location in Barcelona and by age at diagnosis, gender, Breslow logarithm and stage at diagnosis in Essen. Statistically significant results are highlighted in bold.

Table 2. Genetic association of *IRF4* SNP rs12203592 with primary tumour location

	MAF (T allele) *N* (%)		Genotype *N* (%)									
			Present			Other						
Location	Present	Other	CC	CT	TT	CC	CT	TT	OR	95% CI	Adj *p*	FDR Adj *p*
Head and neck	23 (24%)	135 (15%)	28 (58%)	17 (35%)	3 (6%)	320 (72%)	115 (26%)	10 (2%)	**1.79**	**1.07–2.98**	**0.032**	0.096
Trunk	56 (12%)	102 (19%)	171 (76%)	54 (24%)	1 (<1%)	177 (66%)	78 (29%)	12 (5%)	**0.59**	**0.41–0.85**	**0.004**	**0.024**
Upper extremities	18 (16%)	140 (16%)	40 (71%)	14 (25%)	2 (4%)	308 (71%)	118 (27%)	11 (3%)	1.02	0.60–1.75	0.940	0.940
Lower extremities	39 (17%)	119 (16%)	80 (71%)	27 (24%)	6 (5%)	268 (70%)	105 (28%)	7 (2%)	1.13	0.76–1.69	0.550	0.660
Acral	19 (21%)	139 (16%)	21 (61%)	17 (37%)	1 (2%)	320 (72%)	115 (25%)	12 (3%)	1.36	0.80–2.34	0.270	0.406
Mucosal	3 (38%)	155 (16%)	1 (25%)	3 (75%)	0 (0%)	347 (71%)	129 (26%)	13 (3%)	2.90	0.69–12.21	0.170	0.340

Adj *p*: *p* values were adjusted by clinical covariates (age at diagnosis and gender). FDR Adj *p*: *p* values were adjusted by clinical covariates (age at diagnosis and gender) and FDR correction was applied for multiple comparisons. The ORs, CIs and *p* values are based on genotype frequencies. The genetic model used to obtain the OR, 95% CI and Adj *p* values was the log-additive. Statistically significant results are highlighted in bold.
Abbreviation: MAF, minor allele frequency.

regulator receptor (CD5) are involved in melanoma prognosis.[24] This supports the importance of the inherited variation in immune-related genes in modulating melanoma outcome.

As *IRF4* rs12203592 has also been associated with the location of the primary tumor,[13,14] we explored this association in the Barcelona set. In our study, the *IRF4* rs12203592 T allele protected from developing melanoma in the trunk, along the lines of previous reports.[13,14] Furthermore, in our study, the *IRF4* rs12203592 T allele increased the probability of developing melanoma in head and neck, which has not been previously reported. Supporting this finding, a previous study reported that the *IRF4* rs12203592 T allele was associated with melanoma with solar elastosis,[25] which is a characteristic highly prevalent in melanomas developed on the head and neck.[26] We also explored the association between *IRF4* rs12203592 and Breslow thickness which has been previously reported,[27] but this was not detected in our sets of patients. The main limitation of this study is the retrospective design of two sets with different characteristics.

In conclusion, *IRF4* rs12203592 plays a role in the modulation of melanoma outcome, besides melanoma risk. The results of this study also confirm its contribution in the localization of the primary tumour.

Acknowledgement

The work was carried out at the Esther Koplowitz Center, Barcelona. Miriam Potrony is the recipient of a PhD Fellowship from Instituto de Salud Carlos III, Spain; FI14/00231 (PFIS). The research at DS's group is supported by the European Commission under the 6th Framework Programme.

Conflicts of interest

The authors state no potential conflicts of interest.

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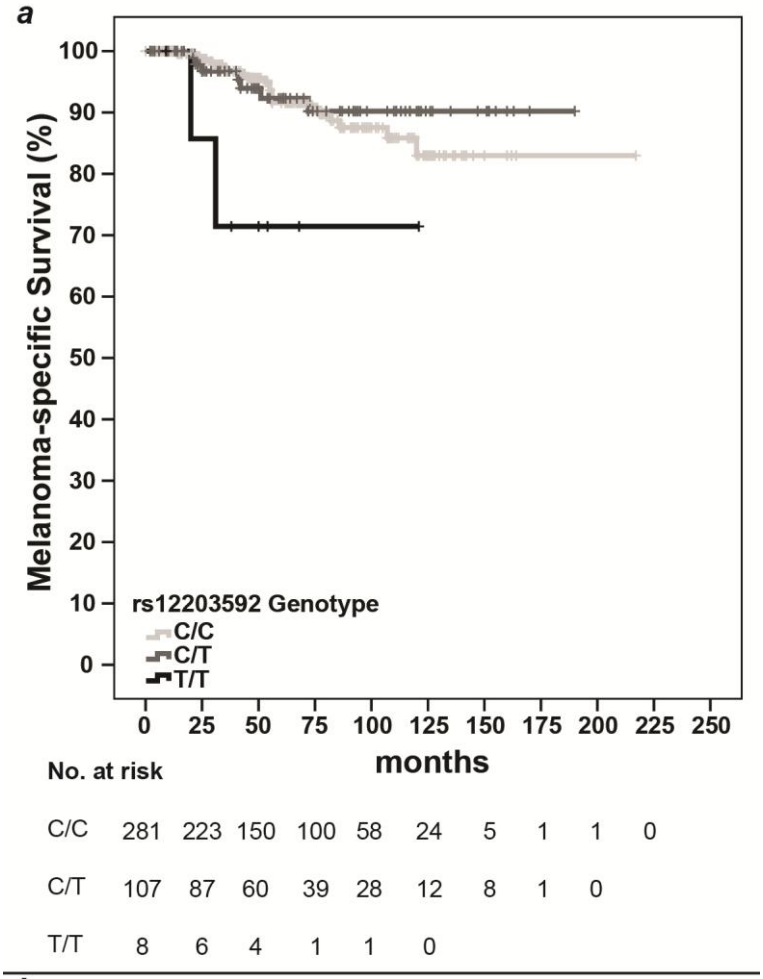
Supporting Information

Figure S1. Melanoma-specific survival curves according to *IRF4* rs12203592 genotype in patients diagnosed before 2011 in Barcelona

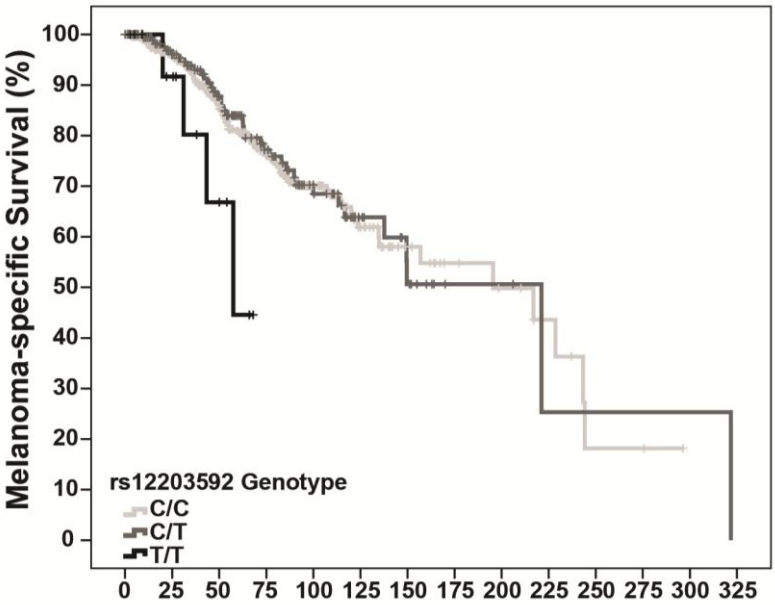


b

Genotype	N	5-year survival rate	HR	95%CI	Adj P
CC	281	0.92	Ref.		
CT	107	0.92	0.87	0.35-2.16	0.762
TT	8	0.71	3.30	0.63-17.23	0.157

a) Kaplan-Meier curves in melanoma patients from Barcelona diagnosed before 2011. Log Rank P-value=0.060. b) Cox Regression analysis results. The Hazard ratio (HR) and P-value were adjusted by age at diagnosis, gender, Breslow logarithm, stage at diagnosis and primary tumor location.

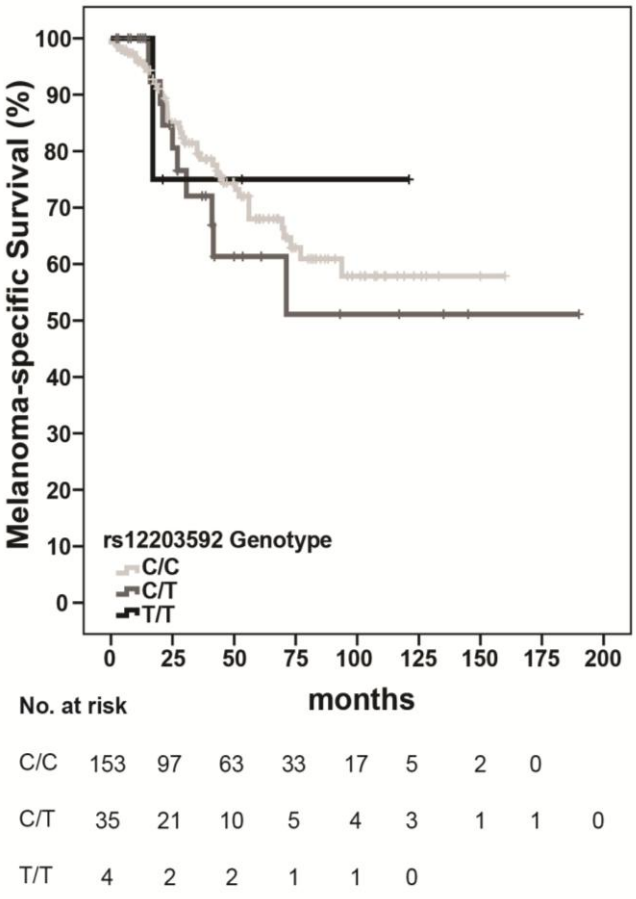
Figure S2. Melanoma-specific survival in Stage I and II patients from both sites together



No. at risk													
C/C	532	400	264	154	85	43	19	12	9	6	2	2	0
C/T	186	137	95	62	39	18	11	2	2	1	1	1	1	0
T/T	15	10	5	0									

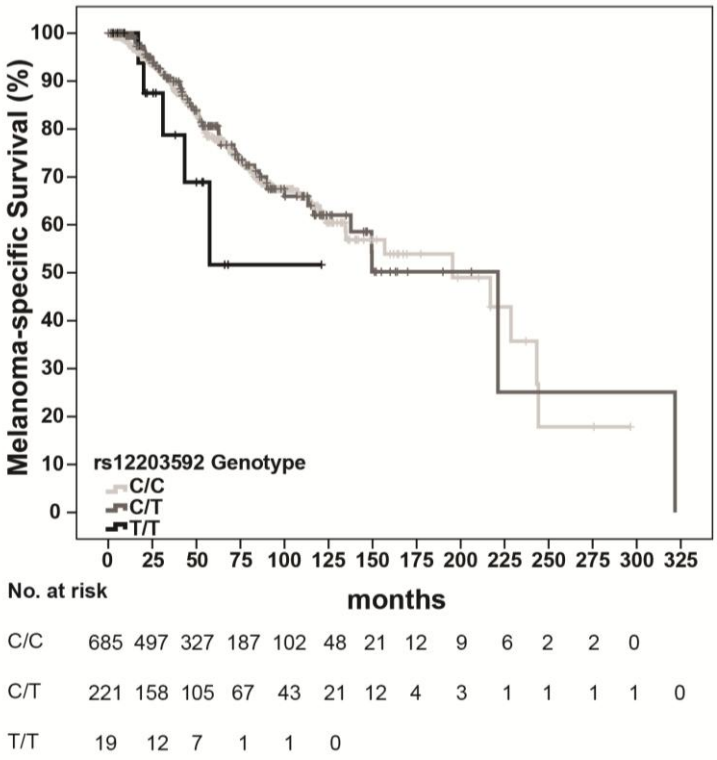
Log Rank p-value=0.104

Figure S3. Melanoma-specific survival in Stage III patients from both sites together



Log Rank p-value = 0.738

Figure S4. Melanoma-specific survival in patients of all stages from both sites together



Log Rank p-value =0.316

Table S1. Survival rate and HR according to different stages in all patients together

Stage	Genotype	N	5-year survival rate	HR	95%CI	Adj P
I and II	CC	532	0.81	Ref.		
	CT	186	0.84	1.04	0.72-1.51	0.849
	TT	15	0.45	3.29	1.20-9.05	**0.021**
III	CC	153	0.68	Ref.		
	CT	35	0.61	1.03	0.51-2.09	0.942
	TT	4	0.75	1.27	0.17-9.50	0.813
ALL	CC	685	0.78	Ref.		
	CT	221	0.80	1.03	0.74-1.43	0.876
	TT	19	0.52	2.20	0.90-5.40	0.084

HR: Hazard ratio. P-value was adjusted by age at diagnosis, gender and Breslow logarithm.

Table S2. Association between the Breslow logarithm and IRF4 rs12203592

	Genotype					
	Mean LogBreslow (No. patients)					
Group	CC	CT	TT	SE	95%CI	*P*
Barcelona	0.28 (348)	0.30 (132)	0.28 (13)	0.01	-0.04,0.06	0.620
Essen	0.40 (337)	0.35 (89)	0.30 (6)	-0.05	-0.11,0.02	0.140

The model used to obtain the SE, 95%CI and P-value was the log additive.

Table S3. Association between the stage and IRF4 rs12203592

		Genotype N(%)			
Group	Stage	CC	CT	TT	*P* *
Barcelona	I	157 (45%)	59 (45%)	6 (46%)	0.596
	II	110 (32%)	50 (38%)	4 (31%)	
	III	81 (23%)	23 (17%)	3 (23%)	
Essen	I	103 (31%)	27 (30%)	3 (50%)	0.333
	II	162 (48%)	50 (56%)	2 (33%)	
	III	72 (21%)	12 (13%)	1 (17%)	

*The Statistic test used was the Fisher Exact Test

ARTICLE 6

Inherited functional variants of the lymphocyte receptor *CD5* influence melanoma survival.

Potrony M, Carreras E, Aranda F, Zimmer L, Puig-Butille JA, Tell-Martí G, Armiger N, Sucker A, Giménez-Xavier P, Martínez-Florensa M, Carrera C, Malvehy J, Schadendorf D, Puig S, Lozano F.

Int J Cancer. 2016;139(6):1297-302.

Aim

To address the putative association between *CD5* allelic variations at SNPs rs2229177 and rs2241002 and clinical outcome of melanoma in two independent cohorts.

Patients

493 melanoma patients from the Melanoma Unit of the Hospital Clinic of Barcelona, Spain (January 1994 – January 2013)

- median time of follow-up: 43 months

215 melanoma patients from University Hospital Essen, Germany (1982 – 2009)

- median time of follow-up: 46 months

Methods

TaqMan Genotyping Assay was used to genotype *CD5* SNPs rs2229177 and rs2241002.

Statistical association and survival analyses.

Variables included in analyses

CD5 SNPs rs2229177 (p.Ala471Val) and rs2241002 (p.Pro224Leu) genotype and haplotype combinations

Age of onset, Gender

Primary melanoma features (Breslow thickness)

Staging information (SLN status)

Time of follow-up and status at last follow-up (alive, dead)

Main results

SNP rs2229177 C allele (p.Ala471) correlated with better melanoma outcome in the Barcelona-cohort (OR = 0.57, 95% CI 0.33–0.99, Adj. *P* = 0.043) and a trend close to statistical significance in the Essen-cohort (OR = 0.63, 95% CI 0.40–1.01, Adj. *P* = 0.051).

SNP rs2241002 T allele (p.Leu224) was associated with increased melanoma associated mortality in both the Barcelona-cohort (OR =1.87, 95% CI 1.07–3.24, Adj. *P* = 0.030) and the Essen-cohort (OR = 1.84, 95% CI 1.04–3.26, Adj. *P* = 0.037).

Pro224-Ala471 haplotype carriers in homozygosis (immune reactive) have a better survival compared with the carriers of the Pro224-Val471 haplotype in homozygosis (immune tolerant) (HR = 0.21, 95% CI 0.07–0.58, Adj. *P* = 0.003).

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International Journal of Cancer

Short Report

Inherited functional variants of the lymphocyte receptor CD5 influence melanoma survival

Miriam Potrony[1], Esther Carreras[2], Fernando Aranda[2], Lisa Zimmer[3], Joan-Anton Puig-Butille[4,5], Gemma Tell-Martí[1,5], Noelia Armiger[2], Antje Sucker[3], Pol Giménez-Xavier[1,5], Mario Martínez-Florensa[2], Cristina Carrera[1,5], Josep Malvehy[1,5], Dirk Schadendorf[3], Susana Puig[1,5] and Francisco Lozano[2,6,7]

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Despite the recent progress in treatment options, malignant melanoma remains a deadly disease. Besides therapy, inherited factors might modulate clinical outcome, explaining in part widely varying survival rates. T-cell effector function regulators on antitumor immune responses could also influence survival. CD5, a T-cell receptor inhibitory molecule, contributes to the modulation of antimelanoma immune responses as deduced from genetically modified mouse models. The *CD5* SNPs rs2241002 (NM_014207.3:c.671C>T, p.Pro224Leu) and rs2229177 (NM_014207.3:c.1412C>T, p.Ala471Val) constitute an ancestral haplotype (Pro224-Ala471) that confers T-cell hyper-responsiveness and worsens clinical autoimmune outcome. The assessment of these SNPs on survival impact from two melanoma patient cohorts (Barcelona, $N = 493$ and Essen, $N = 215$) reveals that p.Ala471 correlates with a better outcome (OR= 0.57, 95% CI = 0.33–0.99, Adj. $p = 0.043$, in Barcelona OR = 0.63, 95% CI = 0.40–1.01, Adj. $p = 0.051$, in Essen). While, p.Leu224 was associated with increased melanoma-associated mortality in both cohorts (OR = 1.87, 95% CI = 1.07–3.24, Adj. $p = 0.030$ in Barcelona and OR = 1.84, 95% CI = 1.04–3.26, Adj. $p = 0.037$, in Essen). Furthermore survival analyses showed that the Pro224-Ala471 haplotype in homozygosis improved melanoma survival in the entire set of patients (HR = 0.27, 95% CI 0.11–0.67, Adj. $p = 0.005$). These findings highlight the relevance of genetic variability in immune-related genes for clinical outcome in melanoma.

Additional Supporting Information may be found in the online version of this article.
M.P., E.C., D.S., S.P. and F.L. contributed equally to this work.
S.P. and F.L. are senior co-authors of this work.
Conflicts of interest: FL is ad honorem scientific advisor at ImmunNovative Developments and inventor of patent EP11382172.2 "New compounds derived from scavenger-like lymphocyte receptors for use in immunotherapy" related to the present work. LZ has served on the advisory boards and received honoraria and travel support from BMS, MSD and Novartis.
Grant sponsor: Spanish Fondo de Investigaciones Sanitarias; **Grant numbers:** 12/00840, 15/00716; **Grant sponsor:** CIBER de Enfermedades Raras of the Instituto de Salud Carlos III (ISCIII), Spain, co-financed by European Development Regional Fund "A way to achieve Europe" ERDF; **Grant sponsor:** AGAUR of the Catalan Government, Spain; **Grant number:** 2014_SGR_603; **Grant sponsor:** European Commission under the 6th Framework Programme; **Grant number:** LSHC-CT-2006–018702 (GenoMEL); **Grant sponsor:** European Commission under the 7th Framework Programme, Diagnoptics; **Grant sponsor:** The National Cancer Institute (NCI) of the US National Institute of Health (NIH); **Grant number:** CA83115; **Grant sponsor:** Fundació La Marató de TV3, Catalonia, Spain; **Grant number:** 201331-30; **Grant sponsor:** Instituto de Salud Carlos III, Spain; **Grant number:** FI14/00231 (PFIS); **Grant sponsor:** Sara Borrell fellowship from Instituto de Salud Carlos III, Spain; **Grant number:** CD15/00016; **Grant sponsor:** Worldwide Cancer Research; **Grant number:** 14-1275; **Grant sponsor:** Fundació La Marató TV3; **Grant number:** 201319-30; **Grant sponsor:** Spanish Ministerio de Economía y Competitividad (Plan Nacional I + D+i, SAF2013–46151-R) co-financed by European Development Regional Fund "A way to achieve Europe" ERDF
DOI: 10.1002/ijc.30184
History: Received 11 Feb 2016; Accepted 2 May 2016; Online 11 May 2016
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Int. J. Cancer: **139**, 1297–1302 (2016)

What's new?

Accumulating clinical and experimental evidence indicates that the T-cell modulatory properties of CD5 might play a role in the antitumor immune response by acting as a putative immune regulator checkpoint. Here, the authors examined the association between *CD5* allelic variations at SNPs rs2229177 and rs2241002 and clinical outcome of malignant melanoma in two independent cohorts. They found that *CD5* functional variants influence melanoma outcome, illustrating the contribution of the genetic variability of the host's immune response on patient survival prospects. This study supports the CD5 immune checkpoint as a new target for the improvement and development of new cancer immunotherapies.

Melanoma is the most deadly of the common skin cancers in Caucasians. To date, histological features of the primary melanoma are important hallmarks of early melanoma prognosis and staging.[1] However, patients within the same tumor stage exhibit a high variation in survival rates suggesting that other intrinsic factors modulate outcome.[2] Melanoma is highly immunogenic, but can evade immune responses *via* several mechanisms including loss of expression of Class I major histocompatibility complex antigens, production of immunosuppressive cytokines, activation of regulatory T-cells and expression of inhibitors for effector T cells (CTLA-4, PD-1/PD-L1).[3] Progress in understanding the relationship between immune and tumor cells has led to the development of successful immunotherapies targeting the so called immune checkpoints (*e.g.*, CTLA-4, PD-L1 and PD-1 inhibitors).[4] Unfortunately, not all patients respond to these therapies and duration and effectiveness vary among responders.[5] Thus, the identification of new therapeutic targets and prognostic biomarkers is essential to further improve patient care.

Accumulating clinical and experimental evidence indicates that the T-cell modulatory properties of CD5 might play a role in the antitumor immune response by acting as a putative immune regulator checkpoint.[6,7] CD5 is a lymphoid-specific 67 kDa receptor, mainly expressed by all T cells and the small B1 subset involved in the production of polyreactive natural antibodies.[8] Even though the nature of the CD5 ligand is still a controversial matter, it is widely accepted that CD5 is indeed a negative regulator of signaling by the clonotypic antigen-specific receptor present on T and B1 cells[9,10] to which it physically associates and co-localizes at the center of the immune synapse.[11] Noteworthy, CD5 is found to be up-regulated in T and B cells with regulatory/suppressor function as well as in T (either CD4+ or CD8+) and B cells anergized by repeated antigen stimulation.[7,8]

No CD5 deficiencies have been reported in humans so far. However, several coding nonsynonymous single nucleotide polymorphisms (SNP) have been identified in the *CD5* gene.[12] Interestingly, the *CD5* gene has been under recent evolutive selective pressure, probably long after the first colonization of East Asia by anatomically modern humans, the nonsynonymous SNP rs2229177 (NM_014207.3:c.1412C > T), coding for an Ala to Val substitution at the cytoplasmic tail of CD5 (p.Ala471Val), being the most probable target for selection. The rs2229177 variants together with another frequent SNP coding for a Pro to Leu substitution at the extracellular region of CD5 (rs2241002, NM_014207.3:c.671C > T, p.Pro224Leu) constitute different haplotypes, one of which (Pro224-Val471) has been positively selected in East Asian populations.[12] Functional analyses reveal that homozygous carriers of the ancestral Pro224-Ala471 (CC) haplotype present higher *in vitro* T cell proliferative responses and a more severe clinical form of Systemic Lupus Erythematosus compared to homozygous individuals for the more recently derived Pro224-Val471 (CT) haplotype.[13] This finding suggests a link between differential regulation of T-cell signaling by CD5 variants and distinct autoimmune disease outcome.

Considering that the immune system plays an opposite role in autoimmune diseases and cancer, we addressed the putative association between *CD5* allelic variations at SNPs rs2229177 and rs2241002 and clinical outcome of melanoma in two independent cohorts (Barcelona $N = 493$ and Essen $N = 215$).

Material and Methods

Design and samples

The retrospective study comprised two independent Hospital Based series of melanoma patients. Recruitment (and therefore blood sampling) took place wherever possible 3–6 months after diagnosis. Patients were included in the study when the following information was available: confirmed alive/death status at last follow-up (melanoma-specific survival), at least one update in follow-up (months) since the date of diagnosis, sentinel lymph node biopsy result (SLN) (positive/negative), gender (male, female), age at diagnosis and Breslow thickness (mm). Exclusion criteria were lack of germinal DNA sample or lack of signed informed consent.

The first set consisted of a Hospital Based series of 493 melanoma patients from the Melanoma Unit of Hospital Clinic of Barcelona, Spain. Patients were diagnosed with melanoma between 1994 and January 2013 (median time of follow-up: 43 months). Cohort disease status was established through the annual review and review of medical notes, from electronic records of the patients with visits every 3–4 months the first 2 years, every 6 months until 5 years and annual until 10 years.

The second cohort comprised a Hospital Based series of 215 melanoma patients from University Hospital Essen, Germany. Patients were diagnosed with melanoma between 1982

and 2009 (median time of follow-up was 46 months). The cohort disease status was established in the same way as for the Barcelona cohort and included an update of lost to follow-up by phone calls.

In the Barcelona cohort, the patient's stage at diagnosis according to AJCC[1] was: 45% Stage I (22 IA, 187 IB, 13 I unknown ulceration status), 33% Stage II (50 IIA, 53 IIB, 17 IIC, 43 II unknown ulceration status) and 22% Stage III (number of positive SLN or presence of micro/macro metastasis was not recorded in our database, thus patients could not be subclassified into IIIA, IIIB or IIIC). In the Essen cohort, the patient's stage at diagnosis according to AJCC was: 28% Stage I (61 IB), 45% Stage II (40 IIA, 33 IIB, 11 IIC, 12 II unknown ulceration status) and 27% Stage III (24 IIIA, 16 IIIB, 5 IIIC, 13 III with unknown number of positive SLN or presence of micro/macro metastasis). The study was approved by the ethical committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

CD5 genotyping

Genomic DNA was obtained from peripheral blood lymphocytes. TaqMan Genotyping Assays were used to genotype *CD5* SNPs rs2229177 (assay number: C___3237272_10) and rs2241002 (assay number: C__25472293_20) according to the manufacturer's recommendations (TermoFisher, Barcelona, Spain). The 7900HT Fast Real-Time PCR System (Applied Biosystems, Bleiswijk, The Netherlands) was used. The results were analyzed using the Applied Biosystems TaqMan Genotyper Software (TermoFisher, Barcelona, Spain).

In the Barcelona set, SNP genotyping was successful for rs2229177 and rs2241002 in 99.4% (490/493) and 99.8% (492/493) of patients, respectively. In Essen, both SNPs were successfully genotyped in 99.5% (214/215) of patients. In the two patient sets both SNPs were in Hardy–Weinberg equilibrium (Barcelona: rs2229177 $p = 0.500$ and rs2241002 $p = 1.000$, Essen: rs2229177 $p = 1.000$ and rs2241002 $p = 0.370$). The two *CD5* SNPs analyzed were not in linkage disequilibrium in either patient set ($D' = 0.597$, r2 = 0.100 and $D' = 0.311$, r2 = 0.018 in Barcelona and Essen, respectively).

Statistical analyses

The main clinical event assessed was melanoma-specific survival. A two-sided Pearson chi-squared test was used for general descriptive analyses for categorical variables. A *t* test was used for general descriptive analyses for continuous variables. The Breslow thickness variable did not follow the normal distribution and was transformed using the logarithm function. The melanoma-specific survival according to different haplotypes was assessed using Kaplan–Meier curves and backward multivariate Cox regression analysis. The hazard ratio (HR) and its 95% CI were calculated. SPSS 17.0 was used to perform descriptive statistical analyses and survival analyses. Genotype and haplotype association analyses were performed using the bioinformatics tool SNPStats (http://bioinfo.iconcologia.net/SNPstats).[14] The odds ratio (OR) and its 95% CI were calculated. Gender, SLN, age at diagnosis and log transformed Breslow were included as covariates in the analyses. Information about Ulceration was not included due to the high number of tumors without this information in the Barcelona set of patients. The tests were considered significant if *p* values or adjusted *p* values as applicable was <0.05.

Results and Discussion

Descriptive analyses of the clinical and genetic variables showed that the characteristics known to be associated with worse melanoma prognosis (male gender, melanoma-death and positive sentinel lymph node) and rs2241002 genotype frequencies were significantly different between the two cohorts (Supporting Information Tables S1 and S2). The association analysis of SNP rs2229177 with melanoma-specific survival (Table 1) showed that the ancestral C allele (p.Ala471) had a statistically significant effect on melanoma outcome in the Barcelona-cohort (OR= 0.57, 95% CI = 0.33–0.99, Adj. $p = 0.043$) and a trend close to statistical significance in the Essen-cohort (OR = 0.63, 95% CI = 0.40–1.01, adj. $p = 0.051$). Additionally, the minor T allele of rs2241002 (p.Leu224) was associated with increased melanoma-associated mortality in both the Barcelona-cohort (OR = 1.87, 95% CI = 1.07–3.24, Adj. $p = 0.030$) and the Essen-cohort (OR = 1.84, 95% CI = 1.04–3.26, Adj. $p = 0.037$).

Haplotype analyses with SNPStats showed that the presence of T alleles for both SNPs (Leu224-Val471 haplotype) was associated with increased risk of melanoma-associated death in the Barcelona-cohort (OR = 2.52, 95% CI = 1.22–5.22, Adj. $p = 0.013$), while in Essen, the presence of the C allele in both SNPs (Pro224-Ala471 haplotype) had a protective role on melanoma survival (OR = 0.49, 95% CI = 0.27–0.90, Adj. $p = 0.022$). Thus, the ancestral *CD5* Pro224-Ala471 haplotype associates with increased melanoma-specific survival, while the more recently derived Leu224-Val471 associates with reduced melanoma survival. As functional analyses have revealed that the ancestral *CD5* Pro224-Ala471 haplotype increases the immune activity compared with the Pro224-Val471 haplotype,[13] we assessed the melanoma-specific survival using Kaplan–Meier and Cox regression analyses, comparing individuals homozygous for each haplotype in the entire patient set (Fig. 1a). We identified that carriers of the Pro224-Ala471 haplotype in homozygosis have a better survival compared with the carriers of the Pro224-Val471 haplotype in homozygosis (HR = 0.21, 95% CI 0.07–0.58, Adj. $p = 0.003$). These individuals also have a better survival compared with other haplotype combinations (HR = 0.27, 95% CI 0.11–0.67, Adj. $p = 0.005$) and also compared with homozygotes for the Leu224-Val471 haplotype (HR = 0.20, 95% CI 0.05–0.80, Adj. $p = 0.022$), the most extreme haplotype combination (Fig. 1b). When AJCC staging was included in the model instead of log transformed Breslow and SLN status, the results were similarly significant (data not shown).

Table 1. Genetic association of the CD5 SNPs rs2229177 and rs2241002 with melanoma-specific survival

	rs2229177 (p.Ala471Val)										
	MAF (C allele)		Genotype Frequency								
			Alive			Dead					
	Alive	Dead	CC	CT	TT	CC	CT	TT	OR	95% CI	Adj. P^1
Barcelona ($N = 490$)	0.48	0.36	0.24	0.48	0.28	0.12	0.47	0.41	**0.57**	**0.33, 0.99**	**0.043**
Essen ($N = 214$)	0.48	0.41	0.24	0.49	0.27	0.14	0.53	0.33	0.63	0.40, 1.01	0.051
	rs2241002 (p.Pro224Leu)										
	MAF (T allele)		Genotype Frequency								
			Alive			Dead					
	Alive	Dead	CC	CT	TT	CC	CT	TT	OR	95% CI	Adj. P^1
Barcelona ($N = 492$)	0.23	0.34	0.59	0.36	0.05	0.47	0.37	0.16	**1.87**	**1.07, 3.24**	**0.030**
Essen ($N = 214$)	0.16	0.25	0.71	0.27	0.02	0.54	0.42	0.04	**1.84**	**1.04, 3.26**	**0.037**

The median (range) time of follow-up in months for Barcelona alive and death patients was 42 (1–217) and 44 (3–124), respectively, and for Essen alive and death patients was 48 (1–177) and 38 (8–244), respectively.
MAF: minor allele frequency; OR: Odds Ratio
Minor allele of rs2229177 SNP was C, while minor allele of rs2241002 was T.
The genetic model used was the log-additive.
The statistically significant results are highlighted in bold.
[1]*p*-values were adjusted by age at diagnosis, gender, log transformed Breslow and sentinel lymph node biopsy result (positive/negative).

As patients from different stages at diagnosis (I, II and III) were included in the study, we performed survival analyses according to the functional Pro224-Ala471 *vs*. Pro224-Val471 haplotypes, grouping patients by staging (Supporting Information Fig. S1). Although, we have not enough power to reach significance, a protective effect on melanoma survival of the Pro224-Ala471 haplotype in homozygosis could be observed in the three stages (Stage I: HR = 0.18, 95% CI 0.21–1.53, Adj $p = 0.117$; Stage II: HR = 0.15, 95% CI 0.02–1.22, Adj. $p = 0.077$, and Stage III: HR = 0.39, 95% CI 0.11–1.43, Adj. $p = 0.154$). Thus, the effect of *CD5* variants on the modulation of melanoma outcome is independent of the stage at diagnosis.

The results represent, as far as we know, the first report on the impact of functional germline variants from an immune-regulatory receptor in melanoma outcome. Previous studies have identified SNPs from several non-immune related genes that impact melanoma prognosis. This is the case for the *GC* gene (rs2282679), linking lower serum levels of vitamin D with increased melanoma-specific deaths,[15] and for the *MC1R* gene,[16] for which loss of function variants up-regulate oxidative stress-related pathways and DNA damage,[17] favoring the apoptosis of damaged cells. SNPs in Nucleotide Excision Repair (NER) and Poly [ADP-Ribose] Polymerase 1 (*PARP1*) genes have also been implicated in melanoma prognosis, showing the relevance of the DNA damage and repair system for tumor survival.[18,19] A similar situation applies to genes from the Hippo pathway, which control cell migration, development and organ sizes in diverse species,[20] as well as genes from the Notch[21] and Fanconi anemia[22] pathways. Related with immunity, SNPs in the interleukin locus and angiogenesis have also been associated with melanoma progression.[23,24]

The presence of immune cells in the tumor microenvironment is known to influence melanoma prognosis.[25] Currently available melanoma immunotherapies target lymphocyte receptors involved in down-regulating T-cell effector functions (*e.g.*, CTLA-4, PD-1 and PD-1 ligand), collectively known as "immune check-points." In accordance with previously published studies,[7] the present association study supports a role for CD5 as a new immune modulatory receptor, which paves the way for improvement of current therapies against melanoma. Indeed, available evidence supports the involvement of CD5 in the regulation of antitumour immune responses. Early mouse studies showed the efficacy of a non-depleting anti-CD5 monoclonal against lymphoid and non-lymphoid tumors.[26] Later reports found that *in situ* sensory adaptation of Tumor infiltrate lymphocytes (TILs) from patients undergoing lung carcinoma involves down-regulation in CD5 surface expression.[6] More recently, studies involving CD5-deficient mice[6] and transgenic mice expressing a soluble form of human CD5[27] showed improved anti-tumor responses using non-orthotopic mouse melanoma models (B16 cells).

In conclusion, the present study illustrates an unprecedented, although predictable, fact: the genetic variability of the host's immune response influences melanoma survival. The results are also in line with a recent observation from our group showing that rs2229177 variants impact the survival response of chronic lymphocytic leukemia patients to conventional chemotherapy regimens.[28] Thus, the identification of new inherited variants in immune-related genes may also be useful to identify patients that are going to respond better to available treatments.

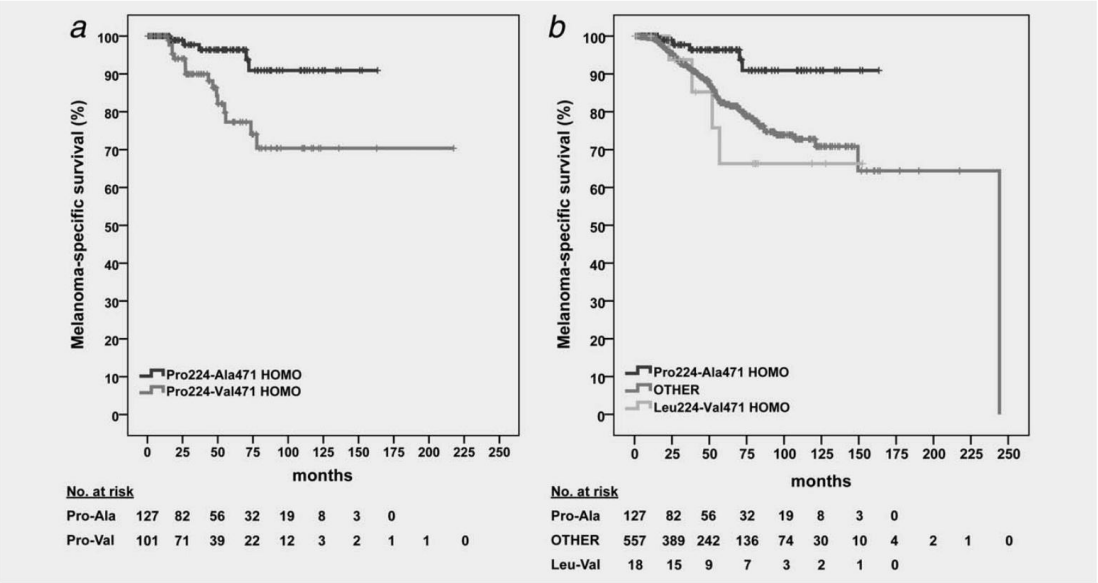

Figure 1. Melanoma-specific survival curve according to the genetic status of *CD5*. (*a*) Melanoma-free survival curve in patients homozygous for the Pro224-Ala471 haplotype (dark gray) *vs*. Pro224-Val471 (light gray). HR comparing homozygous Pro224-Ala471 with homozygous Pro224-Val471 was 0.21 (95% CI 0.07–0.58), Adj. $p = 0.003$. The 5-year survival rate was 0.96 for Pro224-Ala471 homozygous and 0.77 for Pro224-Val471 homozygous. HR and *p*-values were adjusted by age at diagnosis, gender, log transformed Breslow and SLN biopsy result (positive/negative). (*b*) Melanoma-free survival curve in patients homozygous for the Pro224-Ala471 haplotype (dark gray) *vs*. Leu224-Val471 homozygous (light gray) *vs*. other haplotype combination (gray) (Log-rank test $p = 0.010$). HR comparing homozygous Pro224-Ala471 with other was 0.27 (95% CI 0.11–0.67), Adj. $p = 0.005$. HR comparing homozygous Leu224-Val471 with other was 1.22 (95% CI 0.44–3.36), Adj. $p = 0.703$. HR comparing homozygous Pro224-Ala471 with homozygous Leu224-Val471 was 0.20 (95% CI 0.05–0.80), Adj. $P = 0.022$. The 5-year survival rate was 0.96 for Pro224-Ala471 homozygous, 0.82 for other haplotype combination and 0.66 for Leu224-Val471 homozygous. HR and *p*-values were adjusted by age at diagnosis, gender, log transformed Breslow and SLN biopsy result (positive/negative).

Acknowledgements

The authors are indebted to Elena Bosch for helpful critical comments.. The work was carried out at the Esther Koplowitz Center, Barcelona. Miriam Potrony is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain. Fernando Aranda is supported by Sara Borrell fellowship CD15/00016 from Instituto de Salud Carlos III, Spain.

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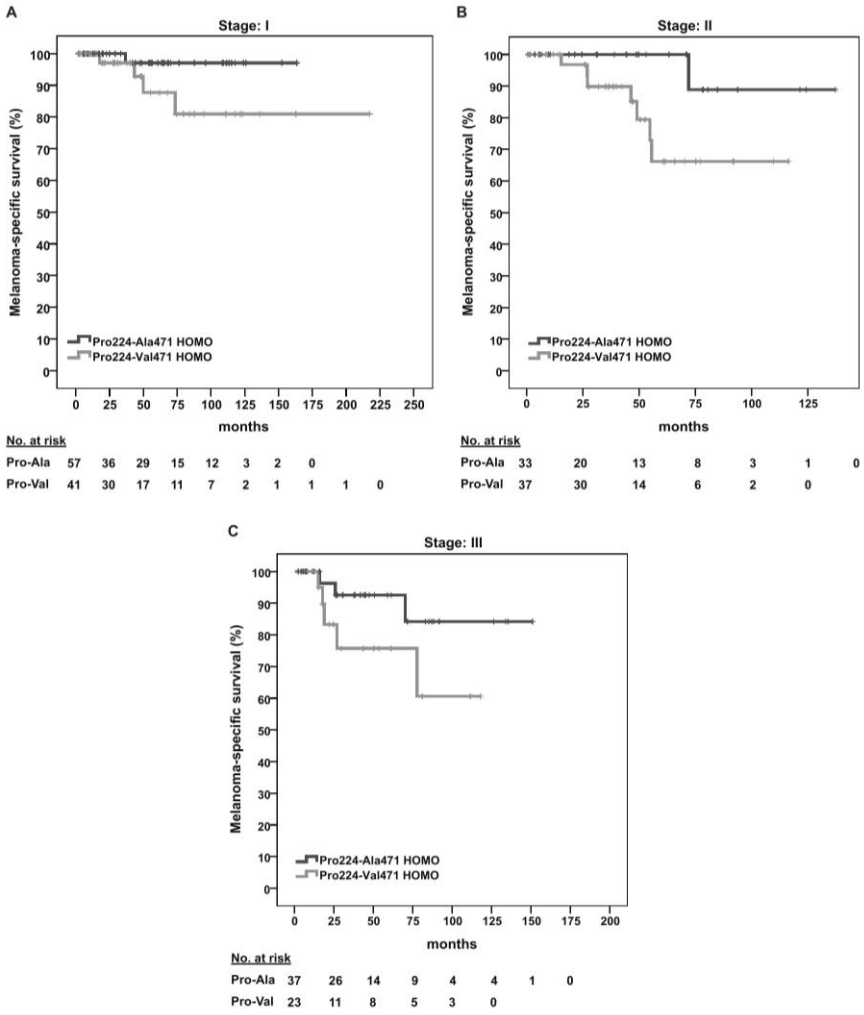
Int. J. Cancer: **139**, 1297–1302 (2016) © 2016 UICC

Supporting information

Figure S1. Melanoma-specific survival curve according to the genetic status of *CD5* by stage at diagnosis



a) Melanoma-free survival curve in stage I patients homozygous for the Pro224-Ala471 haplotype (dark gray) vs. Pro224-Val471 (light gray). HR comparing homozygous Pro224-Ala471 with other was 0.18 (95% CI 0.21 to 1.53), Adj. *P*=0.117. The 5-year survival rate was 0.97 for Pro224-Ala471 homozygous and 0.88 for Pro224-Val471 homozygous. HR and P-values were adjusted by age at diagnosis and gender.

b) Melanoma-free survival curve in stage III patients homozygous for the Pro224-Ala471 haplotype (dark gray) vs. Pro224-Val471 (light gray). HR comparing homozygous Pro224-Ala471 with other was 0.15 (95% CI 0.02 to 1.22), Adj. *P*=0.077. The 5-year survival rate was 1.00 for Pro224-Ala471 homozygous and 0.66 for Pro224-Val471 homozygous. HR and P-values were adjusted by age at diagnosis and gender.

c) Melanoma-free survival curve in stage II patients homozygous for the Pro224-Ala471 haplotype (dark gray) vs. Pro224-Val471 (light gray). HR comparing homozygous Pro224-Ala471 with other was 0.39 (95% CI 0.11 to 1.43), Adj. *P*=0.154. The 5-year survival rate was 0.93 for Pro224-Ala471 homozygous and 0.76 for Pro224-Val471 homozygous. HR and P-values were adjusted by age at diagnosis and gender.

Table S1. Descriptive analysis of the clinical and genetic characteristics of the two patient cohorts studied.

	Barcelona (N=596)	**Essen (N=232)**	
	N (%)	**N (%)**	***P***
Gender			
male	245 (49.7)	126 (58.6)	**0.033**
female	248 (50.3)	89 (41.4)	
Melanoma-specific survival			
alive	460 (93.3)	157 (73.0)	**<0.001**
death	33 (6.7)	58 (27.0)	
SLN biopsy result			
negative	385 (78.1)	141 (65.6)	**0.001**
positive	108 (21.9)	74 (34.4)	
rs2229177 Genotype			
C/C	112 (22.9)	45 (21.0)	0.838
C/T	235 (48.0)	107 (50.0)	
T/T	143 (29.1)	62 (29.0)	
missing	*3*	*1*	
rs2241002 Genotype			
C/C	285 (57.9)	142 (66.4)	**0.039**
C/T	179 (36.4)	67 (31.3)	
T/T	28 (5.7)	5 (2.3)	
missing	*1*	*1*	
	Barcelona (N=596)	**Essen (N=232)**	
	Mean (SD)	**Mean (SD)**	***P***
Age at diagnosis	52.2 (15.1)	53.7 (16.0)	0.231
LogBreslow	0.29 (0.28)	0.38 (0.27)	**<0.001**

SLN: Sentinel lymph node; LogBreslow: log10(Breslow thickness in mm).

Statistically significant p-values are highlighted in bold.

Table S2. Association of the clinical characteristics with melanoma-specific survival

	Barcelona (N=596)			Essen (N=232)		
	Alive	**Death**		**Alive**	**Death**	
	N (%)	**N (%)**	***P***	**N (%)**	**N (%)**	***P***
Gender						
male	222 (90.6)	23 (9.4)	**0.019**	87 (69.0)	39 (31.0)	0.123
female	238 (96.0)	10 (4.0)		70 (78.7)	19 (32.8)	
SLN biopsy result						
negative	368 (95.6)	17 (4.4)	**<0.001**	110 (78.0)	31 (22.0)	**0.025**
positive	92 (85.2)	16 (14.8)		47 (63.5)	27 (36.5)	
	Barcelona (N=596)			**Essen (N=232)**		
	Alive	**Death**		**Alive**	**Death**	
	Mean (SD)	**Mean (SD)**	***P***	**Mean (SD)**	**Mean (SD)**	***P***
Age	51.8 (15.3)	56.8 (12.1)	0.069	54.0 (16.0)	52.8 (16.3)	0.621
LogBreslow	0.27 (0.28)	0.46 (0.34)	**<0.001**	0.36 (0.27)	0.46 (0.27)	**0.014**

SLN: Sentinel lymph node; LogBreslow: log10(Breslow thickness in mm)

Statistically significant p-values are highlighted in bold.

DISCUSSION

CHARACTERIZATION OF KNOWN MELANOMA SUSCEPTIBILITY GENES TO REFINE GENETIC COUNSELING

Articles 1, 2 and 3 were designed to answer to the first goal of this thesis. In **Article 1** we assessed characteristics of families and sporadic MPM harboring *CDKN2A* germline mutations in Spain. We identified *CDKN2A* mutations in 8.5% of sporadic MPM with at least two primary melanomas) and 14.1% of melanoma-prone families with two or more cases of melanoma. More than 70% of the mutations were detected in exon 2 and the most prevalent one was c.301G>T (p.Gly101Trp), the main mutation in the Mediterranean population with a single genetic origin (**Figure 9**).[134]

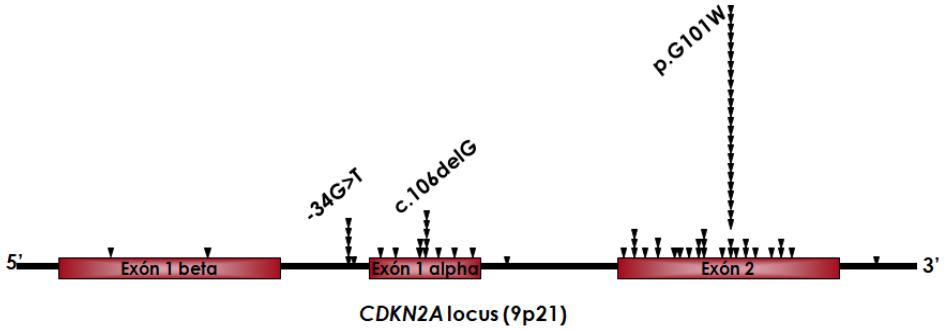


Figure 9. Genetic map of *CDKN2A* mutations in Spanish families

Each inverted triangle represents a pedigree. Most frequent mutations have been listed in the figure.

Consistently with what has been previously reported we identified an increased number of melanoma cases or the number of familial MPM in Spanish *CDKN2A*-positive families, an increased number of primary tumors, and a younger age of onset in *CDKN2A* mutation carriers.[26, 135] A recent publication suggested that the rule of three should be used to recommend genetic testing in France and countries with low to moderate incidence of melanoma, except in families and patients with a first melanoma occurrence before age 40 years in whom the rule of two could be maintained.[136] They analyzed together sporadic MPM and families and detected only 6.5% mutation rate when using the rule of two (≥2 cases of melanoma or of genetically related cancer in the index patient or in his or her first- or second-degree relatives). In our case we detected a higher prevalent mutation rate in both sporadic MPM and families using the same criteria, thus we should consider continuing with the rule of two as an inclusion

criterion. However, it is true that in our article we did not assess how the probability to detect the mutation in patients fulfilling the rule of two changed with the age at diagnosis. Currently, we are including all patients fulfilling this rule, but we should consider a review of this aspect to see whether a specific cut-off of the age of onset should be established to increase the mutation detection rate without losing positive patients.

Interestingly we have identified an increased prevalence of pancreatic, lung and breast cancers among *CDKN2A*-positive families. Pancreatic cancer association with *CDKN2A* germline mutations has been widely reported.[25, 26, 137-142] The role of *CDKN2A* in the risk of developing other cancer types has been less explored. We observed a strong association between the presence of *CDKN2A* mutations and cases of lung cancer within the family. There is a previous study suggesting the association between respiratory cancer and p16-Leiden *CDKN2A* mutation[143] and our group reported a melanoma-prone family in which *CDKN2A* mutations were also present in lung cancer affected individuals.[144] In contrast to the association observed with pancreatic or lung cancer, the statistically significant association with breast cancer was restricted to the analyses combining the information from melanoma-prone families and from those families with sporadic MPM. The risk of breast cancer in *CDKN2A*-positive melanoma-prone families has been reported previously in North-European populations.[139]

The effects of cigarette smoking on lung cancer risk is well documented, but also increases the risk of pancreatic cancer[145] and breast cancer.[146] Pancreatic cancer penetrance is higher in smoker *CDKN2A* mutation carriers than in nonsmoker carriers.[147] Our results indicated that the increased prevalence of these cancers observed in *CDKN2A*-positive families could be explained by genetic factors when they are exposed to the same environmental factors as the general population. Consistent with our findings, a group in Sweden reported simultaneously that *CDKN2A* c.335_337dup (p.Arg112dup) mutation carriers from melanoma-prone families and their first and second-degree relatives had elevated risk for pancreatic, lung, head and neck and gastro-esophageal carcinomas (all of them related to smoking environmental factor).[148] In both studies, we reached the conclusion that genetic counseling in melanoma and cancer prevention programs for *CDKN2A* mutation carriers should be improved and refined based on our observations. Programs should add the recommendation of avoiding

smoking, beyond sun-exposure protection advice and routine total body examination for melanoma early detection.

Individuals carrying *CDKN2A* mutations can also be included in pancreatic cancer imaging surveillance that can detect early-stage surgically resectable pancreatic tumors.[149, 150] In our Hospital, we offer *CDKN2A* mutation carriers the possibility to enter a pancreatic surveillance program with annual echoendoscopic ultrasound after 40 years or 10 years before the age at diagnosis of the youngest pancreatic cancer case in the family. In Catalonia, women from 50 to 69 years old enter an early detection program for breast cancer including mammography every two years. In *CDKN2A*-positive families, we recommend annual gynecologic examination with mammography, starting at the age of 50. Finally, according to the results observed in our study, we offer to ever-smoker *CDKN2A* carriers the possibility to enter a program for early detection of lung cancer using low-dose computed tomography at the age of 50. This screening program has been demonstrated to be effective for early detection of lung cancer.[151] Future studies will have to evaluate the cost-effectiveness of those implementations in the cancer screening for *CDKN2A* carriers.

In **Article 2** we analyzed the prevalence of *MITF* p.Glu318Lys in Spanish patients with melanoma. As *MITF* was suggested to be a medium-risk gene, we decided to include patients with *CDKN2A* mutations affecting p16INK4A (exon 1α and exon 2). We detected a prevalence of 1.9% of the variant in all p16INK4A-wild-type patients, with higher prevalence in the MPM subgroup (2.6%), and a similar one in the p16INK4A-mutated patients (2.9%), while the prevalence in cancer-free Spanish control population was 0.4%. Previous studies identified a prevalence of p.Glu318Lys in melanoma patients that ranged from 1.6% to 2.8%, While in a control population prevalence of this variant was 0.6% in French and Italian populations and 0.8% in UK and Australian populations.[54, 55, 152] In our study *MITF* p.Glu318Lys increased the risk of developing melanoma in a similar magnitude than previous reports (see eTable 2 in the supplementary material provided after the article in the publications section).

Yokoyama and colleagues stated that the presence of this variant was associated with a high nevi count in an Australian and UK population.[55] We also found that *MITF* p.Glu318Lys is associated with a very high nevi count (>200 nevi) in a Mediterranean population. Consistent with the report by Sturm and colleagues,[153] in our study, we

observed that the dermoscopic pattern of nevi present in *MITF* p.Glu318Lys carriers was predominantly reticular. These findings are suggestive of photoinduced nevogenesis[154] and support a role of MITF in nevogenesis.

Ghiorzo and colleagues found an association between *MITF* p.Glu318Lys and the presence of nodular melanomas.[152] Although due to low statistic power we did not confirm such association, we noted two fast-growing melanomas in two *MITF* p.Glu318Lys carriers who were under dermatologic surveillance due to a previous melanoma diagnosis. Dermatologic digital follow-up has been demonstrated to be relevant for detecting melanomas at early stages with a low rate of excisions in patients at high-risk to develop melanoma.[71, 72] During 10 years of dermatologic surveillance of patients at high-risk of melanoma in our melanoma unit (from January 1999 to December 2008), 98 new melanomas were diagnosed in these patients; 54% were in situ melanoma and 46% were invasive melanoma. Among the invasive melanomas diagnosed, none was more than 1 mm Breslow thickness and no melanomas behaved as fast-growing melanomas.[71, 155] Until now in our melanoma unit, the only two fast-growing melanomas identified by dermatologic digital follow-up in individuals at high-risk of melanoma (excluding patients with *Xeroderma Pigmentosum* syndrome, OMIM # 278720) were in *MITF* p.Glu318Lys carriers. Fast-growing melanomas are defined by having a growth rate greater than 0.4 mm per month; while, in general, the melanoma growth rate is approximately 0.1 mm per month and 0.01 mm per month in slow-growing melanomas.[156] A high growth rate is associated with a worse prognosis in melanoma; thus, strategies for early detection of fast-growing melanomas are necessary.[157] To date, *MITF* p.Glu318Lys has been the only germline variant associated with this kind of melanoma.

Thanks to the results of this study we encourage *MITF* p.Glu318Lys carriers to perform monthly total-body self-examination of the skin and they receive fast-track, urgent dermatologic visits if any new lesion appears. Moreover, although *MITF* p.Glu318Lys is considered a medium-risk allele, it also increases the risk of developing RCC. Thus, carriers would also benefit from being included in RCC prevention/surveillance programs. In our Hospital, annual renal ultrasonography is offered starting at the age of 40, as it is a safe and low-cost screening technique to detect the presence of kidney tumor.

We have to keep in mind that the mutation prevalence varies between populations. In another study in which I participated (**Annex 2**[158]) we characterized *CDKN2A* and *MC1R* variants in Latin American population and compared them to the Spanish population. Using the same inclusion criteria, 24% of Latin American families harbor *CDKN2A* mutations, compared to 14% in Spain. Also, a higher prevalence of *MC1R* variants was observed (80.5% in Latin America vs. 67.9% in Spain). This allowed us to establish guidelines for genetic counseling in melanoma in Latin American countries. I also participated in a study in which we characterized the population from a region in southern Switzerland with a high prevalence of melanoma (**Annex 3**[159]). Only one mutation with a founder effect was detected in *CDKN2A* (p.Val126Asp) in 10% of the families assessed but they showed a higher prevalence of *MITF* p.Glu318Lys, identified in 7% of families. Thus, each population has to be properly characterized to be able to offer to their citizens the best personalized genetic counseling.

Finally, in **Article 3** we evaluated the prevalence of *TERT* promoter and *POT1* germline mutations in our *CDKN2A* wild-type families. No mutations were detected in *TERT* in the families irrespective of the *CDKN2A* mutational status. Unfortunately, there are not enough cases with rare germline mutations in *TERT* promoter to assess if there are specific cancer types or traits that are enriched in subjects who carry *TERT* promoter mutations.

Unlike *TERT* promoter mutations, *POT1* germline mutations were present in 1.75% of *CDKN2A*-negative families. Although the number of reported pedigrees with *POT1* mutations is limited, MPM patients are present in multiple pedigrees,[42, 43, 160] including the present study. Moreover, other cancer types occur in the pedigrees. A recent study has identified a *POT1* germline variant in a melanoma-prone family with multiple cases of thyroid cancer and goiter.[160] We identified two melanoma patients in different families carrying *POT1* mutations who also develop thyroid cancer or goiter, thus supporting a role for *POT1* variants in the predisposition of thyroid malignancies. *POT1* germline mutations have also been identified in families with Li-Fraumeni-Like syndrome with cardiac angiosarcoma[161, 162] and chronic lymphocytic leukemia.[163] Nevertheless, more studies should be performed to assess the role of *POT1* in the susceptibility to other cancers.

Thanks to **articles 1, 2 and 3** of this thesis we have been able to improve and refine genetic counseling in melanoma, giving an answer to the first aim of the thesis. **Figure 10** illustrates the past measures and the new improvements made for our patients thanks to our results and supporting works that have been published during this period.

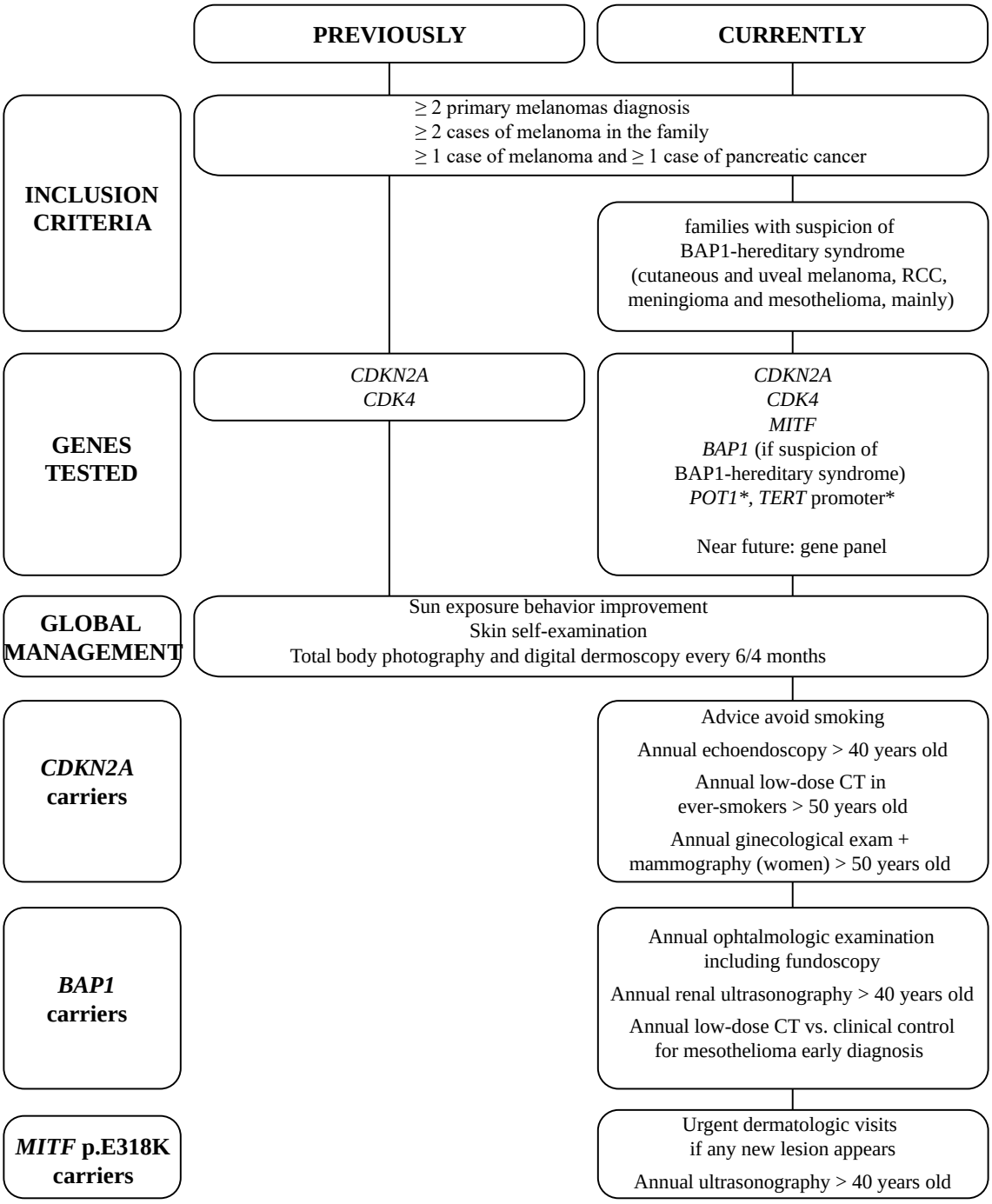


Figure 10. Comparison of genetic counseling offered previous the first thesis article publication in melanoma and the current one

*Those genes were studied for research purposes. Families were only notified when pathogenic variants were detected to offer a better genetic counseling.

Note that for individuals that do not carry germline pathogenic variants dermatological follow-up is offered depending of the family history and also depending on the physical exploration and other phenotypic traits associated with the risk to develop melanoma.

The age indicated for the initiation of other cancer screening is orientative. When patients in the family have developed other cancers younger than the ages proposed, early detection in that family should be considered to start 10 years before the youngest diagnosis of the cancer assessed.

In near future, we will implement NGS techniques for genetic testing such as cancer genes panel, clinical exome, or whole exome sequencing as those methods are more cost-effective than studying the genes one by one. Specific analysis protocols will be used to assess first genes directly involved in melanoma susceptibility and second, if no mutations are detected and the family has a suspicion of other cancer predisposition syndromes, other genes could be assessed.[164] This massive gene screening will lead to the identification of multiple high-risk genes mutated in a family, that now are usually not detected because when a mutation is identified in one gene we do not continue sequencing the others (except for medium-risk genes). This will allow the detection of an increased number of carriers in the families and be able to offer strict dermatologic follow-up to a wider amount of people at risk. For some of the newest high-risk genes, we still do not have enough information to personalize genetic counseling accordingly. We will have to wait a few years until a higher number of families with mutations in those genes will have been detected to assess deeply their features.

Thanks to **Articles 1, 2 and 3** (and **Annex 2**) and all the data generated in our lab during this period of time we have a wider picture of the distribution of germline variants in known melanoma susceptibility genes in our sporadic MPM patients and families. **Figure 11** illustrates the current gene mutation/variant distribution in disease-free control population, sporadic melanoma patients with single melanoma, sporadic MPM, and melanoma-prone families visited at the Melanoma Unit of Hospital Clínic of Barcelona.

In **Annex 7** the equivalent figures from sporadic MPM and melanoma-prone families according to the number of primary tumors or melanoma cases, respectively, is available. As expected we see an increment of the percentage of mutations detected when using the rule of three, but we still have a good mutation rate detection in patients and families with two tumors or cases, respectively.

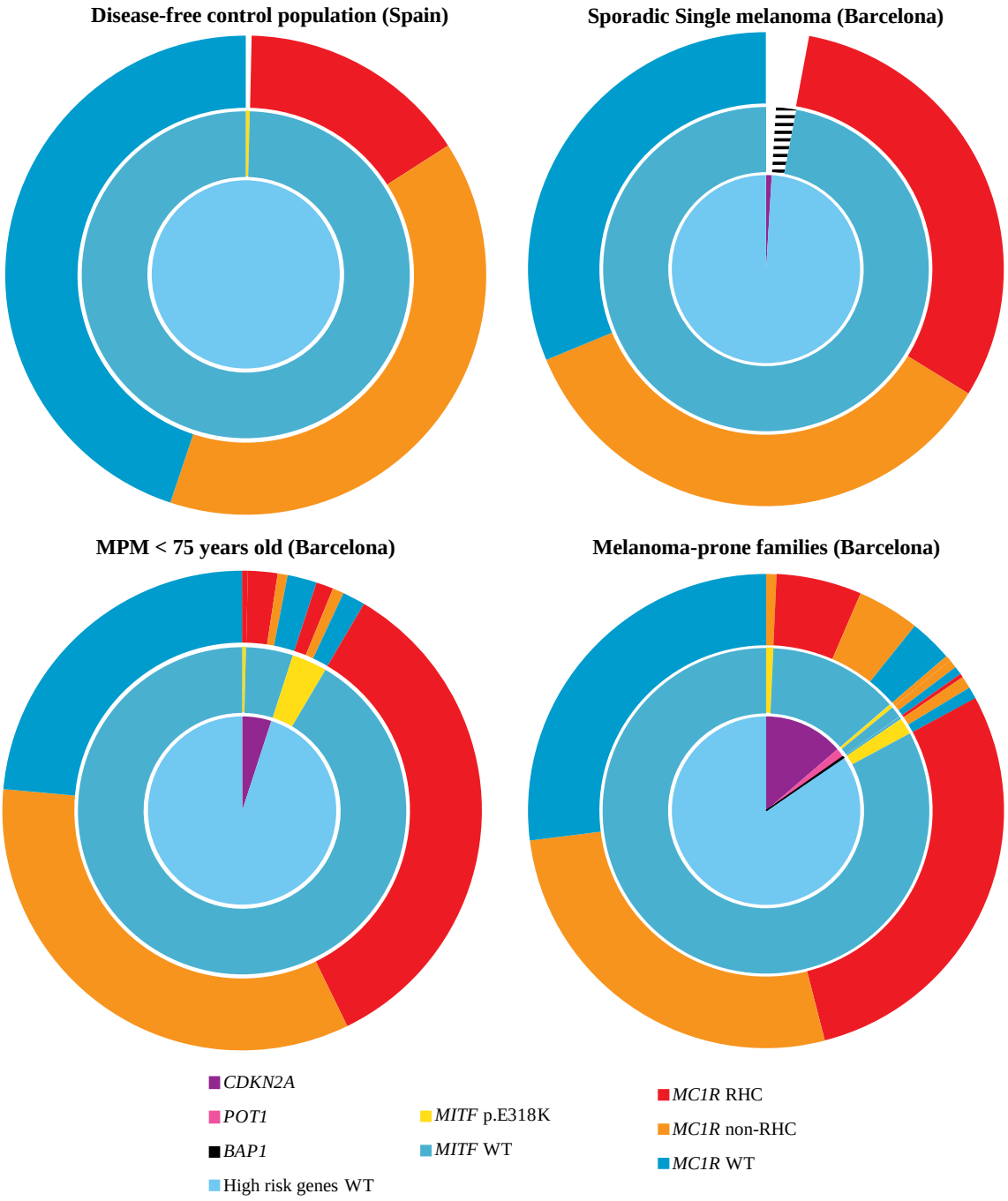


Figure 11. Current distribution of prevalent pathogenic variants according to gene affected in Melanoma

MPM: sporadic multiple primary melanoma patients; WT: wild-type

Each central round represents high-risk genes, the medial circle represents *MITF* status and the external circle represents *MC1R* status. *MITF* and *MC1R* data was not available for a subset of patients (19% and 36%, respectively, in MPM and 50% and 28%, respectively, in families). For the graphical representation, the variant percentage has been extrapolated for *MITF* and *MC1R* missing data, considering that variant proportion will be maintained in each group with the increase of sample size.

For melanoma-prone families, only index cases were considered.

For control population, *CDKN2A* data was obtained from Harland et al. 2014,[165] *MITF* data was obtained from Article 2 and *MC1R* data from and Tell-Martí et al. 2015.[166] For sporadic melanoma patients with

single melanoma, *CDKN2A* data was obtained from Harland et al. 2014,[165] *MITF* data was not available in this set of patients and an estimated proportion was painted with black and white lines, *MC1R* data was obtained from in-house data, partially published in Puig-Butille et al. 2013.[53] For sporadic MPM and melanoma-prone families visited at the Melanoma Unit of Hospital Clínic of Barcelona data derived from articles 1, 2 and 3, Annex 2 and additional sequenced individuals during the last two years in our Unit, including also a family carrying a *BAP1* mutation (data not published).

We can observe that some patients and families carry germline high-risk mutations plus medium-risk variants in *MITF* and/or *MC1R*. The role of genetic modifiers of high-risk variants is poorly understood. To date, studies assessing the modulator effect of genetic variants in *CDKN2A* carriers have been focused on *MC1R*. In fact, those studies have demonstrated that the presence of *MC1R* variants increase the melanoma penetrance in *CDKN2A* carriers and correlate with significant anticipation of melanoma diagnosis.[167, 168] Future studies with larger cohorts will be able to assess the role of *MITF* as a modulator gene or how those genes modulate the penetrance of other high-risk genes.

Nevertheless, as observed in **Figure 11**, even including *POT1* and *TERT* promoter in genetic testing in familial melanoma in Spain, high-risk mutations in melanoma-prone families remain unknown in >80% of families and >90% of sporadic MPM. Thus, there is still a need to identify new genes involved in genetic susceptibility in our region and this need is extended worldwide (**Annex 1**).[18] This brings us to the second goal of this thesis.

IDENTIFICATION OF NEW MELANOMA SUSCEPTIBILITY LOCUS AND FUTURE PERSPECTIVES

Linkage analysis is likely to detect regions containing high-risk variants or genetic features segregating with the disease. In melanoma beyond the first linkage studies that allowed the identification of *CDKN2A* as a melanoma susceptibility gene, few studies have used genome-wide linkage analyses, either using microsatellite marker sets or high-density SNP arrays, to identify new susceptibility loci in *CDKN2A* wild-type melanoma-prone families. These studies have been conducted mostly in pedigrees of Northern European ancestry. These studies identified 1p22, 9q21, and 17p12-p11 as melanoma susceptibility loci.[169-171] Notably, the regions detected in these studies were restricted to each geographic population without overlap between studies and results typically achieved suggestive evidence for linkage. To date, only one study has been

conducted in Mediterranean melanoma pedigrees from Italy, which failed to detect results with suggestive or significant linkage evidence.[172]

In **Article 4**, in response to the second goal of this thesis, we report the results of a genome-wide linkage screen performed on 11 melanoma-prone families in which we detected significant linkage to the 11q14.1-q14.3 locus for melanoma susceptibility. Although the number of families included in the study is lower than in previous studies, the subset of families was enriched by the inclusion of highly informative families since 54.4% families had ≥4 melanoma cases.

A previous GWAS study performed in melanoma patients reported a melanoma locus at the 11q14.3 region. The study detected the strongest evidence of association near rs1393350 encompassing *TYR* gene, which plays a key role in human pigmentation and is a low-risk melanoma gene.[173] In our study, the two subregions with strongest linkage evidence within 11q14.1- q14.3 did not include the *TYR*, suggesting that, other than pigment related alleles in the *TYR* gene, more melanoma susceptibility genes are present in this genomic region.

The *DLG2* (Discs Large MAGUK Scaffold Protein 2), *PRSS23* (Protease, Serine 23), *FZD4* (Frizzled Class Receptor 4), and *TMEM135* (Transmembrane Protein 135) genes were located in the regions with the strongest linkage evidence. The biological information about this set of genes is limited, but they are all plausible candidates for cancer susceptibility.[174-177] However, further sequencing data and molecular studies are necessary to elucidate the possible role of these genes in melanoma susceptibility.

We have detected seven additional loci with suggestive linkage evidence. Interestingly one of these loci overlaps the 3q29 locus that has previously been detected in *CDKN2A* wild-type Swedish families.[178] The finding of a common region in independent studies from countries with a different genetic background, strongly suggests that this region may contain genetic factors associated with familial melanoma susceptibility. The overlap region contains plausible candidates involved in proliferation and apoptosis, lipid transport, serin/threonin phosphatase PP1 inhibition, or Notch activation.[179-182]

In our study, we included a *CDKN2A*-positive family in which two melanoma cases did not carry known high-risk nor medium-risk melanoma susceptibility variants. Both patients developed early onset melanomas (age of 32 and 33 respectively), similar to the *CDKN2A*-positive members in the family (median age: 36 years old, range 18-63). A

study assessing phenocopies in *CDKN2A*-positive families in the US and Sweden found a median age of onset of 46 years old in wild-type patients within the family, which was around 10 years older than the median age of onset of their *CDKN2A*-positive cases.[183] As our patients fulfilled the characteristics to suspect of the presence of a high-risk gene, we thought it was worth it to try to identify new genes also in this family. We detected three loci with suggestive linkage evidence indicating that, in addition to the *CDKN2A* mutations, other genetic factors underlie the increased melanoma risk observed in the members of this family.

There is still a high missing heritability for familial melanoma. Studies such as **Article 4** are needed to provide clues to new genomic regions to focus on, in order to identify new high-risk variants that may explain part of the missing heritability of melanoma susceptibility. Future NGS studies or candidate gene targeted sequencing from these regions may allow the identification of new genetic factors implicated in melanoma susceptibility.

To date, most NGS studies focused on the identification of new high-risk melanoma susceptibility genes are based on whole-exome sequencing (WES) data.[43, 184, 185] Recently WES in two pedigrees with evidence of 9q21 linkage has allowed the identification of a rare non-synonymous variant in *GOLM1* (Golgi Membrane Protein 1) which was proposed as a new candidate gene for melanoma susceptibility.[186] However, no other gene has been proposed as a good candidate in any other of the previously detected loci.

In general, we have been focused on studying coding variants, but maybe part of the missing heritability is explained due to non-coding variants affecting splicing and regulatory elements including promoters and enhancers. Furthermore, studies have suggested that enhancers may be organized in a higher-order structure within regulatory units, which are called super-enhancers.[187] Understanding how this super-enhancers work would be useful to enlighten some gaps in cell differentiation and tumorigenesis. Moreover, understanding the mechanisms underlying the manner in which these super-enhancers influence genetic programs, could also help us understand part of the missing heritability.[188, 189] Another element to take into consideration is expression quantitative trait locus (eQTL), which by definition is a genome sequence variant that results in gene expression changes. Now, eQTLs are prime suspects in the search for contributions to

the causality of complex traits.[190] However, we have to keep in mind that eQTL can regulate distant genetic elements,[191] thus much information is still needed on that perspectives.

With WES, deep intronic variants that can alter splicing or expression are lost, as well as regulatory non-coding variants. The use of whole-genome sequencing (WGS) gives us more information as non-coding variants are captured. There are some tools that allow us to predict the probably pathogenic effect either by focusing on evolution conservation such as GERP – Genomic Evolutionary Rate Profiling[192] – or tools combining multiple information such as CADD – Combined Annotation-Dependent Depletion – method.[193] Nevertheless, functional studies will be needed at the end to prove pathogenicity of the variant or even combine with whole RNA (Ribonucleic acid) sequencing (RNA-seq) that can detect novel splicing variants for particular genes or fusion transcripts.[194] We also need to keep in mind that a few families can carry large genomic deletions that will need the implementation of specific bioinformatic pipelines to be able to detect them correctly.[195] Alternatively, we could use comparative genomic hybridization or other methods allowing the identification of gain/loss material. Thus, reanalyses of NGS data already generated or sequencing of non-coding regions (in combination with RNA-seq if considered necessary) may reveal new candidate genes or even new structural and non-coding pathogenic variants in the genes already described to be associated with melanoma susceptibility.

Characterization of new susceptibility genes allows the identification of population at-risk to develop melanoma and we can offer them a better dermatologic surveillance to detect melanomas at early stages, which is the best way to influence on disease prognosis and melanoma survival. However, 90% of melanomas are developed by sporadic patients with genetic burden mainly due to low or medium-risk variants. Identification of such population is not yet well established. Independent studies have assessed how polygenic risk scores could predict melanoma risk. Using information from 16 to 21 SNPs associated with melanoma detected in GWAS studies, polygenic risk scores only detects barely two-fold increased risk comparing the group with highest genetic risk with the one with lowest genetic risk.[196-198] Even combining the information from the available low-risk genes into polygenic scores, it does not allow us to discriminate efficiently between sporadic people at high or low risk alone. Still, if we

add the genetic information into a phenotypic risk model, it achieves a modest improvement in risk prediction.[198, 199] Nevertheless, today the only way to promote early diagnostic in this group is by public skin cancer prevention campaigns.

IDENTIFICATION OF MELANOMA PROGNOSTIC GENES

Once a melanoma patient is diagnosed, we currently classify them using the AJCC staging system and we establish a specific follow-up protocol for each stage. As explained in the introduction this staging system only takes into consideration some clinicopathological features: Breslow thickness, ulceration, presence of SLN metastasis, regional nodal metastasis, satellitosis, in transit metastasis or distant metastasis.

In our Hospital patients stage IIB, IIC and III are included in intensive monitoring for early detection of recurrence. The follow-up protocol consist of: a) periodic consultations (every 3 months during the first two years, every 6 months during the 3rd to 5th year, and annually up to the 10th year) performed by a dermatoncologist with physical examination of the skin including palpation of lymph nodes and the primary scar, dermoscopy, and digital dermoscopy when needed; b) laboratory tests scheduled with the same frequency as visits, and c) total body computed tomography and brain magnetic resonance performed every 6 months from the diagnosis until the 5th year, and then just an annual chest X-ray up to the 10th year. This follow-up protocol allows the detection of early relapses.[200]

Early diagnosis of metastasis may improve the capability to respond to treatments, based on results of a study in which low tumor burden correlates with higher response to immunotherapy.[201] However, a study of cost-effectiveness showed that computed tomography scan was cost-effective during the first 4 years, while brain magnetic resonance was cost-effective only during the first year.[202] It is known that radiation from imaging techniques used for relapse surveillance increases the risk to develop tumors.[203] If we are able to identify correctly patients with a very low probability of relapse within the same clinical stage, we can avoid the radiation that they will receive during the existing protocol follow-up. Thus, there is a need to improve the classification of patients into different prognosis groups to adapt their follow-up strategies and made the system more cost-effective.

Molecular information from the tumor, in melanoma, is taken into consideration for the therapy treatment selection in metastatic patients. Therefore, patients with *BRAF* mutations in amino acid Valine 600 are candidates to receive anti-BRAF therapies while wild-type *BRAF* patients will not benefit from that therapeutic strategy. There have been attempts to develop algorithms that consider molecular expression data from the tumor to better predict the patient survival in early stages of the disease. In other cancer types, such as breast cancer, multigenic molecular signatures have already been incorporated into clinical practice, to some extent.[204] In melanoma, at least two companies have released a test with their own signature to predict relapse in early stages melanomas.[205-207] However, those tests have not been incorporated to the clinical practice, yet.[208]

Another important player in prognosis is the genetic background of the individual but to date, this information is not included in any follow-up guideline. A few studies have attempted the identification of genetic factors acting as modulators for the melanoma outcome. Most studies are focused on the study of candidate genes or pathways and the effect of each gene is very low. This leads us to the third goal of the thesis. Our findings in **Articles 5 and 6** suggest that finding good scores incorporating germline variants could improve personalized follow-up.

There exist a tight relationship between genes involved in melanoma susceptibility and genes involved in melanoma prognosis as previously introduced in this thesis. In **Article 5** we assessed the role in melanoma prognosis of a known melanoma low-risk variant (*IRF4* rs12203592) that is also associated with nevi count.[209, 210] Nevi count is the main phenotypic risk feature for melanoma and at the same time, an increased number of nevi has been associated with good prognosis.[99] Ribero and colleagues suggest that this association could be explained because the genetic determinants for nevi count may be associated with biological differences in the tumor.[99] Consistent with the fact that the *IRF4* rs12203592 T is associated with a low nevi count in adults,[209] in our study the *IRF4* rs12203592 T allele confers a worse melanoma prognosis. Thus, our results suggest that *IRF4* may be one of the genes explaining the association between the nevus count and melanoma prognosis.

IRF4 (Interferon regulatory factor 4) encodes a transcription factor expressed in melanocytic and immune system cells lineage.[211] *IRF4* rs12203592 affects a

melanocyte-specific enhancer regulator. The T allele impairs the function of this enhancer, leading to a reduction of *IRF4* expression. MITF and TFAP2A bind this element when the C allele is present, activating IRF4 expression in melanocytes.[212] IRF4 activates the melanogenesis pathway by the regulation of *TYR* expression, together with MITF (**Figure 12a**). This could be one explanation of why these genes are both implicated in nevi count and why IRF4 *rs12203592* is also highly associated with the tanning ability.[213]

It has been suggested that *IRF4* expression enables suppressive regulatory T cells (Treg) to suppress effector T cells.[214] In lymphocytes, contrary to melanocytes, the C allele of *IRF4* rs12203592 inhibits the expression of this gene.[215] Thus, individuals carrying the T allele, have a higher expression of *IRF4* in lymphocytes. Tregs from these individuals may have a higher ability to inhibit the immune response against the tumor, explaining why these individuals have a worse melanoma prognosis (**Figure 12b**).

Considering that at least part of the implication of *IRF4* in prognosis can be explained due to its role in the regulation of immune system, in **Article 6** we assessed the role of functional variants in a new proposed immune checkpoint: *CD5* (CD5 Molecule). CD5 is a lymphoid-specific receptor, mainly expressed by all T cells and B1 cells.[216] CD5 is indeed a negative regulator of signaling by the clonotypic antigen-specific receptor present on lymphocytes.[217, 218] This receptor is up-regulated in T and B cells with regulatory/suppressor function as well as cells anergized by repeated antigen stimulation.[216, 219] *CD5* gene has been under recent evolutive selective pressure. The rs2229177 (p.Ala471Val) variants together with the rs2241002 (p.Pro224Leu) constitute different haplotypes, one of which (Pro224-Val471) has been positively selected in East Asian populations.[220] Functional analyses reveal that homozygous carriers of the ancestral Pro224-Ala471 haplotype present higher in vitro T cell proliferative responses. Furthermore, in patients with Systemic Lupus Erythematosus, a chronic autoimmune disease that can cause severe fatigue and joint pain, homozygous carriers of the Pro224-Ala471 haplotype present a more severe clinical form (lupus nephritis) compared to homozygous individuals for the more recently derived Pro224-Val471 haplotype.[221]

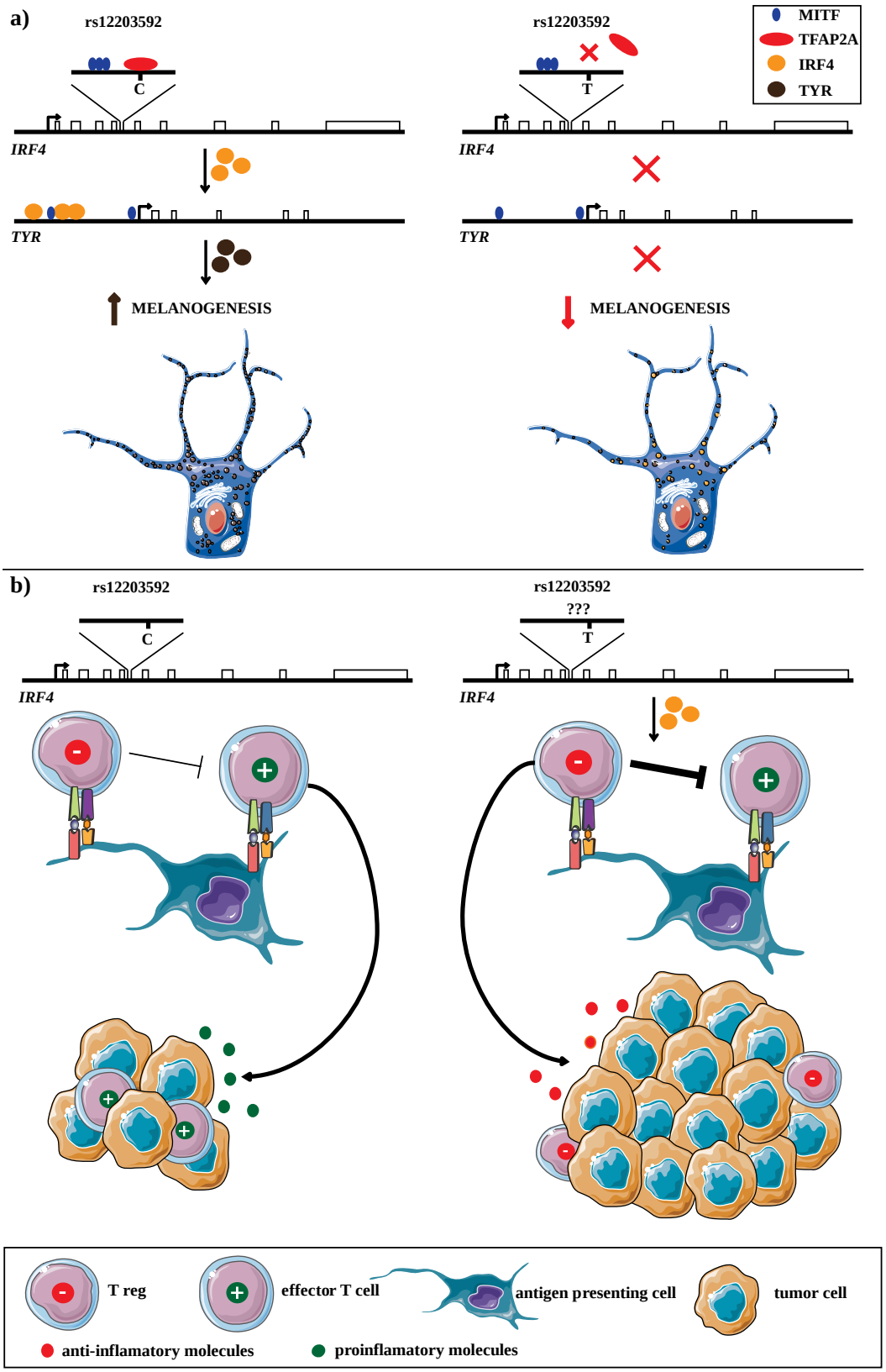


Figure 12. Role of *IRF4* rs12203592 in melanocytes and lymphocytes

a) Role in melanocytes. Adapted from the graphical abstract of reference[212]. With the presence of T allele, *IRF4* is less expressed. Thus it cannot activate *TYR* expression and consequently, the melanogensis

pathway is down-regulated. b) Role in lymphocytes. Although the exact regulation in lymphocytes is less understood, when the T allele is present, *IRF4* is up-regulated, favoring the T regulatory cell (T reg) ability to suppress effectors T cell. Thus the immune system is less active. In that scenario, tumors are more likely to grow and go unnoticed by the immune system.

In our study, we identified that the ancestral *CD5* Pro224-Ala471 haplotype, which correlates with a higher immune activity, was associated with increased melanoma-specific survival, compared with the Pro224-Val471 haplotype, which correlates with a more immune tolerant phenotype. These individuals also have a better survival compared with other haplotype combinations and also compared with homozygotes for the Leu224-Val471.

Tumor and autoimmune diseases are two sides of the same coin when it refers to immunity. In this case, having a more active immune system worsens the outcome in autoimmune diseases and vice versa, a more immune tolerant host will have less autoimmune reactivity but will fight less against the tumor (**Figure 13**). Focusing on functional variants that already play a role in autoimmune diseases could allow the identification of new variants modulating melanoma prognosis.

Current available melanoma immunotherapies target lymphocyte receptors involved in down-regulating T cell effector functions (e.g., CTLA-4, PD-1, and PD-1 ligand). In accordance with previously published studies,[219] the present association study supports a role for CD5 as a new immune modulatory receptor, which paves the way for improvement of current therapies against melanoma. Indeed, available evidence supports the involvement of CD5 in the regulation of antitumor immune responses. Early mouse studies showed the efficacy of a non-depleting anti-CD5 monoclonal against lymphoid and non-lymphoid tumors.[222] Later reports found that *in situ* sensory adaptation of TILs from patients undergoing lung carcinoma involves downregulation in CD5 surface expression.[129] More recently, studies involving CD5-deficient mice and transgenic mice expressing a soluble form of human CD5 showed improved antitumor responses using non-orthotopic mouse melanoma models.[223, 224] All this information together with our work suggest that CD5 could be a good candidate as a target for immunotherapy.

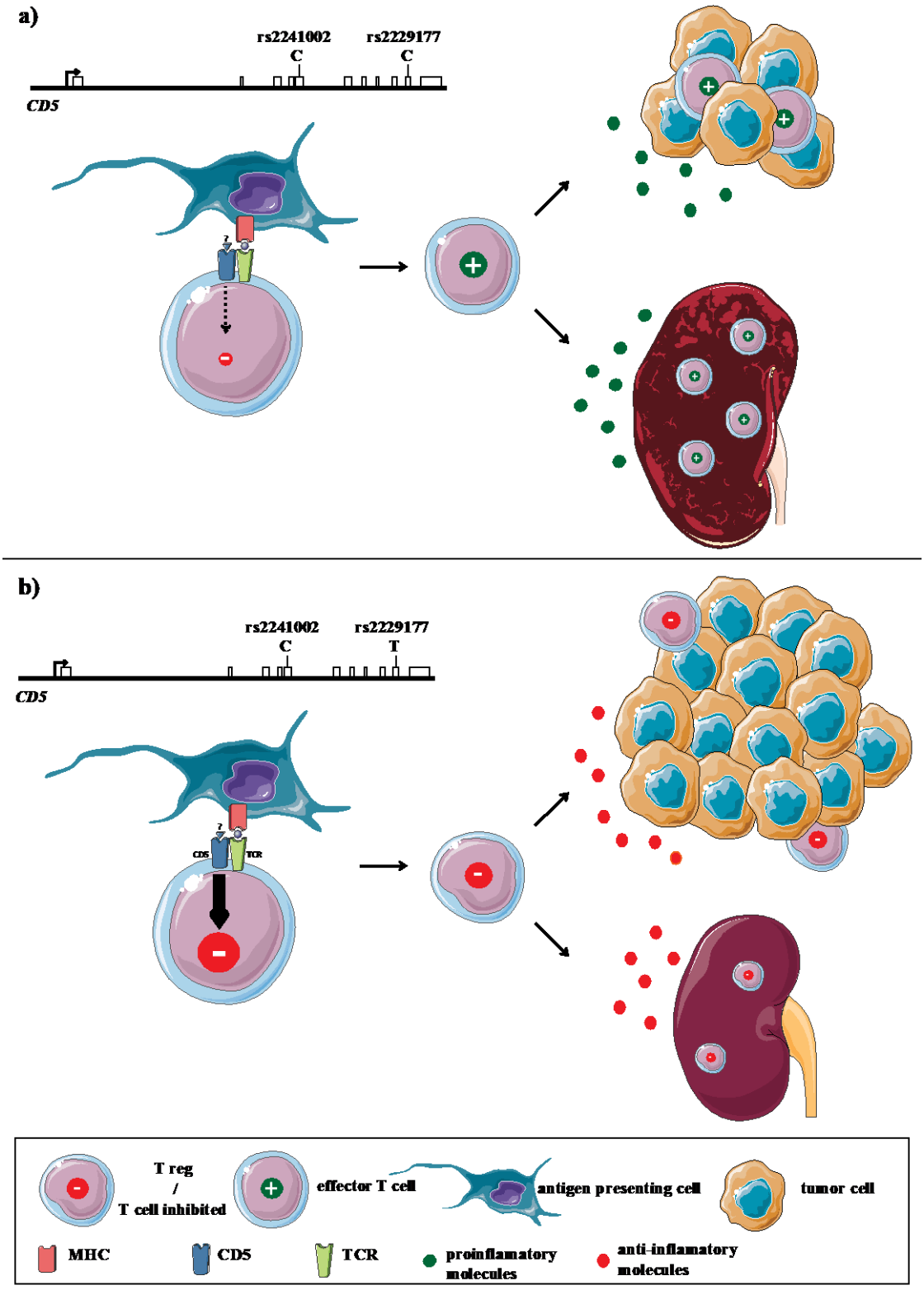


Figure 13. Role of *CD5* variant rs2229177 in cancer and autoimmune diseases

a) The presence of the rs2229177 C allele correlates with a lower activity of CD5 and a higher immune reactivity. This favors the fight against tumor and worsens the phenotype of patients with Systemic Lupus Erythematosus (development of kidney nephritis). b) The presence of the rs2229177 T allele correlates with a high activity of CD5 and a more immune tolerant profile. This favors tumor growth and patients with autoimmune disease have less severe phenotype (for example non-affection of the kidney).

In **Articles 5 and 6** we confirmed that germline variants regulating the immune system can modulate melanoma outcome. In both studies, the variants associated with an immune-tolerant phenotype were associated with a worse melanoma prognosis. This shows that individuals with a more active immune system are in general more able to fight the tumor cells and can eliminate better them conferring a better prognostic to the patient. Thus, future studies should focus on the identification of new inherited variants in a wider spectrum of immune-related genes to be able to create scores able to classify patients into immune-active vs. immune-tolerant at the moment of diagnostic. That way we could reactivate the immune system in the immune-tolerant patients, for example using adjuvant immunotherapy, a therapeutic strategy that has already shown to improve melanoma progression-free survival time in stage III patients free of disease.[225, 226] Furthermore knowing key genes that can be modulating the immune system based on functional germline data generated, such as CD5, may also be useful to design new strategies for immunotherapy development. Finally, we could even use this information to test whether those variants modulating immune system activity are also able to predict patients that are going to respond better to the available treatments, to personalize more the therapeutic strategies.

CONCLUSIONS

1. We have established the genetic bases of melanoma susceptibility in our population and refined genetic counseling, knowing the mutation prevalence of each gene and adapting secondary prevention measures in melanoma and other tumors according to the genetic testing results.
2. *CDKN2A* mutation carriers, besides sun protection advice and dermatologic surveillance, should receive recommendations on avoiding smoking and can be included in early detection programs for pancreatic, lung and breast cancers.
3. *MITF* p.Glu318Lys carriers should be given fast-track visits to dermatology as they may be at risk to develop fast-growing melanomas and can be included in early detection programs for renal cancer.
4. *POT1* is mutated in a subset of melanoma families in Spain, thus genetic testing in melanoma should include the analysis of this gene.
5. Genome-wide linkage analysis in melanoma-prone families from Spain has allowed the identification of a new locus at 11q involved in familial melanoma.
6. We have identified new genes modulating melanoma outcome based on the study of candidate genes involved in melanoma susceptibility, nevi count, and immune regulation.
7. *IRF4* rs12203592 T functional variant, associated with a low melanogenesis (and low nevus count) and immune tolerance, correlates with a worse melanoma survival.
8. Inherited functional variants of the lymphocyte receptor *CD5*, associated with more immune reactivity, correlates with better melanoma outcome.

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ANNEXES

Annex 1

Additional Article 1

Title: Update in genetic susceptibility in melanoma.

Authors: Potrony M, Badenas C, Aguilera P, Puig-Butille JA, Carrera C, Malvehy J, Puig S.

Publication: Ann Transl Med. 2015;3(15):210. Doi: 10.3978/j.issn.2305-5839.2015.08.11. (Review)

Journal not indexed in JCR. The manuscript has >20 cites (WOS, SCOPUS).

Contribution of the doctoral student to the article: The doctoral student participated in the recruitment of information from the literature and was responsible for writing the original draft of the manuscript.

Review Article

Update in genetic susceptibility in melanoma

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Contributions: (I) Conception and design: C Badenas, S Puig; (II) Collection and assembly of data: All authors; (III) Manuscript writing: M Potrony, C Badenas, JA Puig-Butille, S Puig; (IV) Final approval of manuscript: All authors.

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Abstract: Melanoma is the most deadly of the common skin cancers and its incidence is rapidly increasing. Approximately 10% of cases occur in a familial context. To date, cyclin-dependent kinase inhibitor 2A (*CDKN2A*), which was identified as the first melanoma susceptibility gene more than 20 years ago, is the main high-risk gene for melanoma. A few years later cyclin-dependent kinase 4 (*CDK4*) was also identified as a melanoma susceptibility gene. The technologic advances have allowed the identification of new genes involved in melanoma susceptibility: Breast cancer 1 (BRCA1) associated protein 1 (*BAP1*), *CXC* genes, telomerase reverse transcriptase (*TERT*), protection of telomeres 1 (*POT1*), *ACD* and *TERF2IP*, the latter four being involved in telomere maintenance. Furthermore variants in melanocortin 1 receptor (*MC1R*) and microphthalmia-associated transcription factor (*MITF*) give a moderately increased risk to develop melanoma. Melanoma genetic counseling is offered to families in order to better understand the disease and the genetic susceptibility of developing it. Genetic counseling often implies genetic testing, although patients can benefit from genetic counseling even when they do not fulfill the criteria for these tests. Genetic testing for melanoma predisposition mutations can be used in clinical practice under adequate selection criteria and giving a valid test interpretation and genetic counseling to the individual.

Keywords: Melanoma; melanoma susceptibility; familial melanoma; genetic counseling; cyclin-dependent kinase inhibitor 2A (CDKN2A); cyclin-dependent kinase 4 (CDK4); breast cancer 1 associated protein 1 (BAP1); protection of telomeres 1 (POT1); telomere

Submitted Jul 27, 2015. Accepted for publication Aug 03, 2015.
doi: 10.3978/j.issn.2305-5839.2015.08.11
View this article at: http://dx.doi.org/10.3978/j.issn.2305-5839.2015.08.11

Introduction

Melanoma is the most aggressive of the common skin cancers, being responsible for 75% of deaths from skin cancer (1). Melanoma incidence is rapidly increasing especially in Caucasian populations (1,2). Although development of melanoma during childhood is rare, it can appear at any age and is the second most diagnosed cancer among patients under 30 years old (3). For this reason, melanoma is one of the cancers with more years of productive life lost (4). If melanoma is diagnosed in its early stages, it can be cured by surgical removal. However, when the diagnosis is delayed, melanoma is the tumor with the highest metastatic capacity, since it increases by 10% per millimeter of thickness. Despite the improvement in survival of metastatic patients thanks to new targeted therapies, diagnosis and treatment of the initial tumor remains the best strategy for dealing with melanoma (5). Thus, the identification of individuals at high risk of developing melanoma is essential to reduce melanoma mortality, as prevention and early detection programs can be implemented.

Melanoma etiology is complex and heterogeneous as it involves environmental, phenotypic and genetic risk factors. The main environmental risk factor for melanoma is the exposure to ultraviolet radiation

(UVR) (6). UVR causes DNA damage through the formation of pyrimidine dimers, photoproducts, gene mutations, oxidative stress, inflammation and immunosuppression, favoring the carcinogenic process (7). UVR has been widely demonstrated to be implicated in nevo-genesis and melanoma-genesis. UVR can induce clinical changes (increased pigmentation, scale formation and erythema) as well as dermoscopic changes in pigmentation (changes in size and number of globules and dots, regression features such as bluish gray granules, blurred pigmented network and increased vascularity) (8-10). The use of sunscreen can prevent part of the UVR effects on nevi (11). Furthermore, a 10-year follow-up study showed that the daily use of sunscreen reduces the melanoma detection rate, suggesting that regular sunscreen use may prevent melanoma development (12). However, there are intrinsic risk factors that can predispose to melanoma. Phenotypic characteristics such as red or blond hair, blue or green eyes, fair skin with low tanning ability, freckles, multiple melanocytic nevi (100 or more) or 5 or more atypical nevi are associated with an increased risk to develop melanoma (13,14). Personal history of melanoma increases 5-8% the risk of developing a second melanoma (15,16). Finally, family history of melanoma has been widely associated with an increased melanoma risk (14).

Familial cancer syndromes are recognized for gathering high-risk features, including a cluster of relatives within the family who have the same or similar cancers as the patient, the development of cancer at a young age, the presentation of more than one synchronous or metachronous primary tumor or more than one tumor within a specific group of tumors that are features of a syndrome (17). Approximately 5-10% of melanoma cases occur in a familial context (18). In these families, melanoma susceptibility is inherited following an autosomal dominant inheritance pattern with incomplete penetrance (19). However, multiple primary melanoma patients also have inherited melanoma susceptibility (20).

This review aims to give an overview of the melanoma susceptibility genes known to date and genetic counseling in melanoma.

Melanoma susceptibility genes

High risk genes

Melanoma high risk genes are defined as genes that when mutated in an individual confer a high risk of developing melanoma and are usually associated with multiple melanoma cases within the family. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) was the first gene associated with melanoma susceptibility. The *CDKN2A* gene is located in the 9p21 locus and encodes two tumor suppressor proteins p16INK4A and p14ARF via differential splicing and alternative reading frames. The protein p16INK4A, encoded by the α-transcript (composed by exon 1α, 2 and 3), promotes the arrest of the cell cycle in the G1 phase by inhibiting RB (retinoblastoma protein) phosphorylation through cyclin-dependent kinase 4 (CDK4). The β-transcript (composed by exon 1β, 2 and 3) encodes p14ARF and acts through the p53 pathway inducing the cell cycle arrest or favoring apoptosis (21). Furthermore, both p53 and p16INK4A play an important role on cell damage response and senescence (22). In 1992, Cannon-Albright and colleagues described for the first time 9p21 as a familial melanoma locus thanks to linkage analyses (23). Two years later the first germline mutations in *CDKN2A* were reported in familial melanoma (24). Many studies have been performed since then trying to investigate the role of *CDKN2A* in melanoma susceptibility. To date, *CDKN2A* is the main high risk gene involved in melanoma susceptibility (25). Mutations in that gene are found in around 20% of melanoma-prone families [*Figure 1*, references (25-39)], but the *CDKN2A* mutation frequency can range from 5% to 72% depending on the selection criteria used and the geographical areas (26,40). The *CDKN2A* mutation detection rate increases with the number of cases in the family. Mutations in *CDKN2A* are also detected in sporadic multiple primary melanoma patients (SMP). The *CDKN2A* mutation frequency in SMP with at least two primary melanoma is around 9% (20,27,41). As in familial studies, the probability to detect mutations in SMP increases with the number of total primaries. On the other hand, the probability to have *CDKN2A* mutations in sporadic melanoma patients without personal or familial history of melanoma is about 1% (42). The penetrance for melanoma in *CDKN2A* carriers varies between geographical areas and increases with age. Bishop and colleagues reported that at the age of 50, the melanoma penetrance for carriers was 13% in Europe, 50% in the US and 32% in Australia, whilst at the age of 80 the penetrance was 58% in Europe, 76% in the US and 91% in Australia (43). Furthermore, individuals

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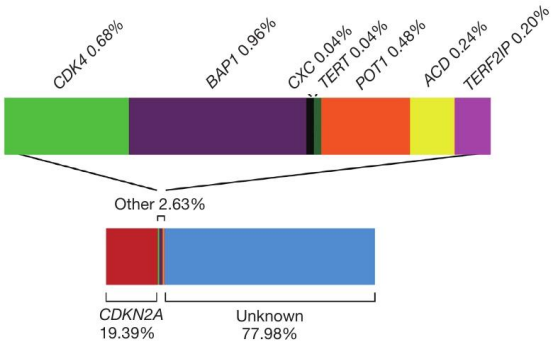

Figure 1 The figure represents the prevalence of mutations in high-risk melanoma susceptibility genes identified to date in melanoma-prone families. This figure includes the genetic information of 2,511 pedigrees: 487 *CDKN2A*, 17 *CDK4*, 24 *BAP1*, 1 *CXC*, 1 *TERT*, 12 *POT1*, 6 *ACD* and 5 *TERF2IP* mutated pedigrees respectively, and 1,958 families with unknown mutation. The data used to prepare this figure was that reported in previous research articles (25-39). We have excluded information from the Goldstein *et al.*'s study (26), from the groups belonging to GenoMEL consortium that have published updated reports of *CDKN2A* mutations alone, to avoid duplicates. For all those groups that have reported different updates of their families over time, we have selected the most recent article or the one that included a greater number of families. CDKN2A, cyclin-dependent kinase inhibitor 2A; CDK4, cyclin-dependent kinase 4; BAP1, Breast cancer 1 (BRCA1) associated protein 1; TERT, telomerase reverse transcriptase; POT1, protection of telomeres 1.

carrying *CDKN2A* germline mutations have an inherited risk to develop other cancer types beyond melanoma. A strong association between *CDKN2A* germline mutations and pancreatic cancer has been reported (27,40,44-48). Families with *CDKN2A* mutations also have an increased risk to develop breast, lung and other tobacco-related cancers (27,47,48).

CDK4 was the second high risk melanoma susceptibility gene identified (49). *CDK4* is an oncogene located within the 12q14 chromosomal region and encodes a protein that controls cell cycle progression through the G1 phase. To date mutations in this gene have been described in 17 melanoma-prone families and in all of them the mutation affects the same amino acid (Arginine 42) (50). This amino acid is located in the p16INK4A binding domain of the CDK4 protein. Thus, when *CDK4* is mutated, p16INK4A cannot inhibit the CDK4 kinase activity resulting in the progression of the cell cycle. *CDK4* mutation carriers phenotypically behave similarly to p16INK4A mutation carriers (50). This is consistent with the functional impact that mutations in both genes have at the cellular level, which results in the activation of the same pathway.

Breast cancer 1 (BRCA1) associated protein 1 (*BAP1*) germline mutations have been associated with a cancer syndrome characterized by the presence of broad tumor types: cutaneous melanoma, uveal melanoma, mesothelioma, renal cell carcinoma (RCC), atypical Spitz tumors, atypical intradermal tumors (MBAITs) and multiple basal cell carcinomas (51-65). However, the whole tumor spectrum associated with germline *BAP1* mutations is still unknown. *BAP1* is located in the chromosomal region 3p21 and encodes a protein that plays a tumor suppressor role through transcription regulation by chromatin remodeling and the ubiquitin-proteasome system. BAP1 is a deubiquitylase that participates in multi-protein complexes that regulate key pathways including the cell cycle, cell differentiation, cell death, gluconeogenesis and the DNA damage response (54). The frequency of *CDKN2A* wild-type melanoma-prone families with mutations in *BAP1* is not well established, but beyond cutaneous melanoma, families bearing *BAP1* mutations seem to be enriched by uveal melanoma, mesothelioma, RCC, other cutaneous tumors and other cancers.

Copy number variants (CNV) assessed genome wide, allowed the identification of a duplicated region on 4q13 segregating with melanoma in one melanoma-prone family (66), suggesting that some melanoma-prone families seem to carry mutations in private genes. The whole duplicated region contains 10 genes, most of them belonging to a family of CXC chemokines, such as melanoma growth-stimulating activity α (*CXCL1*) and interleukin 8 *(IL-8*). Both genes have been shown to stimulate melanoma growth *in vitro* and *in vivo* (66).

The most recent findings in melanoma susceptibility involve genes that play a role in telomere maintenance. Telomeres consist of tandem nucleotide repeats (TTAGGG) and are located at the ends of chromosomes. The telomerase enzyme, the shelterin protein complex and many other accessory proteins are also comprised in the telomeres. They maintain genomic stability and chromosomal integrity by protecting chromosome ends from degradation, end-to-end fusion, and atypical recombination (67). Telomeres shorten both with age and following exposures associated with cancer risk, such as

smoking and ultraviolet (UV) irradiation (68,69). Thus, telomere maintenance processes are natural candidates for explaining carcinogenesis (70). Horn and colleagues identified a germline mutation in the promoter of telomerase reverse transcriptase (*TERT*) in a melanoma-prone family using multipoint linkage analyses and target-enriched high-throughput sequencing. *TERT* is located in 5p15 and encodes the catalytic subunit of the telomerase, which is the ribonucleoprotein complex that maintains telomere length (71). Recently, two independent groups identified rare germline variants in protection of telomeres 1 (*POT1*) in 12 *CDKN2A* wild-type melanoma-prone families using next generation sequencing (72,73). *POT1* is located within the 7q31 chromosomal region and encodes a protein of the telomeric shelterin complex. POT1 plays an important role in telomere maintenance by preventing the inappropriate processing of the exposed chromosome ends, caused by pathways related to DNA damage response, and regulating telomerase function (74). Furthermore, Aoude and colleagues described germline mutations in melanoma-prone families located in two more genes involved in the shelterin complex, *ACD* and *TERF2IP*. This study included 510 melanoma-prone families without mutations in the known melanoma susceptibility genes to date. They identified six families with mutations in *ACD* and four families with *TERF2IP* mutations. The mutations were segregating with melanoma in the families (75). Overall, the germline mutations in genes that play a role in telomere maintenance (*TERT*, *POT1*, *ACD* and *TERF2IP*) may explain around 1% of familial melanoma cases, showing the relevance of telomere maintenance in melanoma susceptibility (*Figure 1*).

Figure 2 shows the interaction and pathways implicated until now in familial melanoma susceptibility.

Low to moderate risk genes

Melanocortin 1 Receptor *(MC1R)* is considered a moderate risk gene and its role in melanoma susceptibility has been widely studied. *MC1R*, located in 16q24, is one of the master regulator genes in human pigmentation and encodes the α melanocyte-stimulating hormone (α-MSH) receptor 1. *MC1R* is a highly polymorphic gene in the Caucasian population. Variants in *MC1R* have different functional effects, either at the level of α-MSH binding or cAMP signaling, resulting in changes in the ratio between eumelanin (brown pigment) and pheomelanin (red-yellow pigment, potentially mutagenic) ratio (76). *MC1R* variants are associated with skin and hair pigmentation and, independently of their phenotypic effect, *MC1R* variants are associated with an increased risk of developing melanoma (77). When the MC1R function is highly compromised, this usually results in the red hair color phenotype (RHC). The most common *MC1R* variants have been classified as r variants, when there is a low association with RHC (p.V60L, p.V92M, p.R163Q) and R variants, when they are highly associated with RHC (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H) (78,79). The R variants are those most implicated in melanoma susceptibility. The melanoma risk conferred by R variants varies from two times risk per R allele in the general population to three times risk, in the familial melanoma context. The risk is additive, thus carriers of two R alleles have a 4 to 6 times increased risk than individuals without these variants. Studies assessing the modulator effect of *MC1R* variants in *CDKN2A* carriers demonstrate that the presence of *MC1R* variants increase the melanoma penetrance in *CDKN2A* carriers (80). The r variant p.R163Q is associated with increased risk of melanoma in high sun exposed geographic areas (81) and with a subtype of melanoma associated with chronic sun damage, lentigo maligna melanoma (82).

Microphthalmia-associated transcription factor *(MITF)* is also considered a moderate risk gene. Two independent groups identified the rare functional variant in *MITF* p.E318K (rs149617956) that increases melanoma risk and also predisposes to RCC (83,84). *MITF* is located in the chromosomal region 3p14 and is a master regulator gene of melanocyte development and differentiation, and it is also associated with melanoma development and progression (85). *MITF* p.E318K occurs at a conserved SUMOylation position and this variant decreases the number of SUMO-modified MITF forms. As SUMOylation of MITF represses its transcriptional activity, p.E318K increases the MITF transcriptional activity and may result in the up-regulation of distinct sets of genes. Furthermore, this variant promotes invasive and tumorigenic behaviors in melanoma and RCC cells and might favor a phenotypic switch of melanoma cells towards a tumor-initiating cell phenotype (84). The prevalence of p.E318K in melanoma patients ranges from 1.6% to 2.8%, whilst in a control population prevalence of this variant is 0.6% in French and Italian populations and 0.8% in UK and Australian populations (83,84,86). Furthermore, this variant has been associated with phenotypic features such as high nevi count, fair skin and non-blue eye color (83,87,88).

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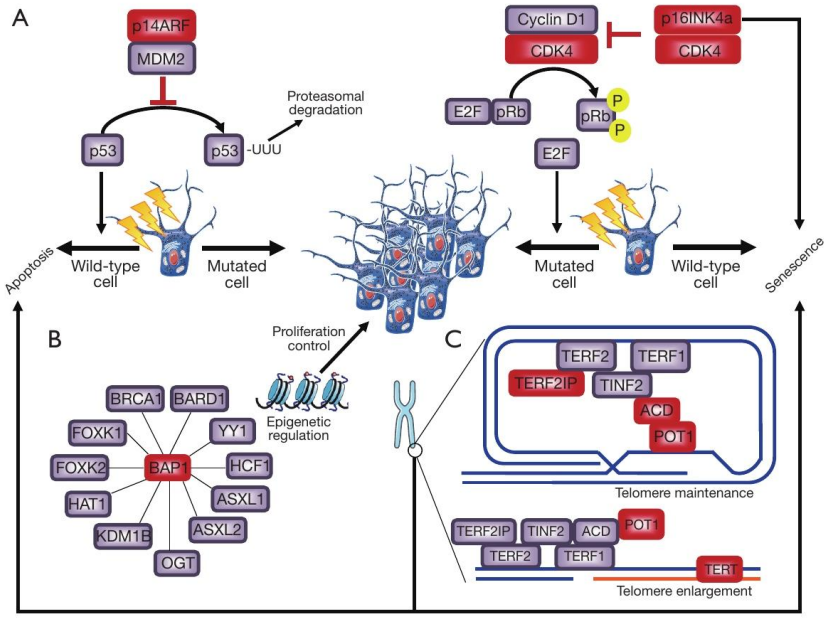

Figure 2 Cell biology functions and pathways of the genes involved in melanoma susceptibility. In red all proteins encoded by known high risk melanoma susceptibility genes. (A) *CDKN2A* encodes two different proteins p16INK4a and p14ARF. p16INK4a is an inhibitor of the cyclinD1/CDK4 complex and induces the arrest of the cell cycle. When p16INK4a can perform its function correctly, if a cell is damaged it can induce senescence. However when *CDKN2A* is mutated, the cyclinD1/CDK4 complex is not inhibited and phosphorylated pRb, releasing E2F transcription factor that can induce a cell cycle progression. If a cell is damaged the release of E2F can favor an aberrant proliferation that can lead to tumor development. In the same way, when CDK4 is mutated, p16INK4a cannot interact with it and cannot inhibit the release of E2F. p14ARF in front of a damaged cell induces apoptosis through the p53 pathway. If p14ARF is mutated, MDM2 can promote p53 ubiquitination and p53 is degraded in the proteasome. Thus when a cell is damaged, the lack of activation of p53 can allow that cell to avoid apoptosis (21). (B) BAP1 is involved mainly in epigenetic and gene transcription regulation interacting with multiple partners (54). When *BAP1* is mutated it cannot properly do its activity and this can lead to a dysregulation of gene transcription that can alter the proliferation controls. BAP1 has a tumor suppressor role, thus when a cell is damaged, mutant BAP1 cells can start proliferating in an aberrant way that can lead to the development of a tumor. (C) Telomeres form a protective structure at the ends of the chromosome (T-loop) that is covered by the shelterin complex (TERF2IP, TERF1, TERF2, TINF2, ACD and POT1). This complex also mediates the interaction between telomeres and the telomerase (TERT). Shelterin binds to the telomere through TERF1 and TERF2. POT1 binding to the single-stranded DNA overhang prevents access of telomerase to telomeres. When POT1 is unbound, the telomerase is able to extend telomeres. Furthermore when the telomeres are unprotected by the shelterin complex senescence and apoptosis can be induced. Mutations in genes lead to telomere dysregulation and can be involved in the development of a tumor when the cells are damaged (67-69,72-75). CDKN2A, cyclin-dependent kinase inhibitor 2A; CDK4, cyclin-dependent kinase 4; BAP1, Breast cancer 1 (BRCA1) associated protein 1; POT1, protection of telomeres 1.

Other genes containing common variants in the population show association with melanoma, but the risk conferred by these common variants is low. Each variant alone does not reach a two-fold increase melanoma risk (89). These genes are involved in different biological processes. There is a group of genes involved in the nevi count and pigmentation including: agouti signaling protein (*ASIP*) encoding an antagonist of α-MSH, Tyrosinase (*TYR*) encoding a protein implicated in eye color determination and tanning ability, Tyrosinase-related protein 1 (*TYRP1*) encoding a protein that stabilizes TYR, Oculocutaneous albinism II (*OCA2*) playing a role in eye color and pigmentation (90), Methylthioadenosine phosphorylase (*MTAP*) also implicated in human pigmentation and

paired box 3 (*PAX3*) involved in face and eye development and also associated with nevi count (91). There are also genes involved in the immunologic system such as some interleukins (IL-10, IL-1β), tumor necrosis factor alpha (*TNF-α*), human leukocyte antigen (*HLA*) class II genes (92) or interferon regulatory factor 4 (*IRF4*) (93). Another gene group is related with metabolism: cytochrome P450 family 2 (*CYP2D6*) that plays a role in the lipid metabolism, genes encoding glutathione transferases (*GSTM1*, *GSTT1* and *GSTP1*) (94), fat mass and obesity associated (*FTO*) encoding a protein related with the non-heme iron enzymes (95) and Vitamin D receptor (*VDR*) involved in mineral metabolism. Poly ADP-ribose polymerase 1 (*PARP1*), which encodes a chromatin-associated enzyme that modifies nuclear proteins, is also associated with melanoma (93).

Low to moderate risk genes have a weak impact on melanoma susceptibility and families with these variants usually have one or occasionally two melanoma cases. But if a combination of low to moderate variants is inherited, more melanoma cases could be present in the family. However, as UV radiation is the main environmental risk factor for melanoma, families with low to moderate risk variants living in areas with an increased UV radiation could have more melanoma cases (96).

Melanoma genetic counseling and management

Genetic counseling and risk assessment is the process of identifying and counseling individuals at increased risk of developing cancer, and distinguishing between those at high risk (high penetrance genes/families), those at a moderate increased risk (multifactorial etiology or low to moderate penetrance alleles), and those at average risk (97). This information can also be used to assess other at risk individuals within the family. Genetic counseling is offered to melanoma-prone families to better understand the meaning of the disease and genetic susceptibility, the inheritance pattern, the option of genetic testing, the understanding of all the possible results and the primary and secondary prevention of melanoma as well (96). The process includes melanoma risk assessment, the possibility of genetic testing, informed consent, disclosure of test results and psychosocial assessment as in other cancer genetic counseling or assessment (96,97). Doctors should refer patients with a personal and/or family history of melanoma, with features suggestive of having an increased melanoma risk, to a melanoma genetic assessment unit. Genetic counseling often implies genetic testing, but all patients can benefit from genetic counseling, even if they are not candidates for genetic testing. A broad number of reports highlight that genetic counseling in cancer is useful for the screening and management of patients (97). Genetic counseling aims to help patients and families. Genetic counselors should inform patients about the genetic risk factors for melanoma and other cancers, the indications for genetic testing, the chance of detecting a mutation, the possible reports (positive, negative or inconclusive), the possible implications for other family members, provide psychological assessment, and also offer recommendations for cancer screening and UV protection and how these might change with testing (96). Although mutations can be detected in these patients, families and patients negative for a familial mutation still have up to a two-fold greater risk of developing melanoma than that of the general population, due to other melanoma susceptibility genes and environmental factors shared between families (43,98).

To date, in melanoma genetic counseling, genetic testing is mainly focused on *CDKN2A* and *CDK4*. However, its use in clinical practice has been controversial due to the variation in the estimates of *CDKN2A* mutation penetrance (28-91%) depending on the study design, the ethnic background, UV exposure and co-inheritance of low to moderate predisposing variants (such as *MC1R* variants) (99). It is also difficult to assess patients because individuals negative for the mutation segregating in the family are still at increased risk of melanoma, compared with the general population. Genetic testing for melanoma predisposition mutations can be used in clinical practice under adequate selection criteria and giving a valid test interpretation and genetic counseling to the individual. People should understand that the interpretation of test results is difficult and the potential impact on clinical management is limited. Candidates for genetic testing should only be individuals with at least a 10% chance of carrying a mutation before the test is performed. These individuals should belong to melanoma-prone families or families with melanoma-related cancers (sarcoma, early onset breast cancer, brain tumors or pancreatic cancer) and/or have multiple primary melanomas. Patients with an early age at onset or with multiple or atypical nevi do not fulfill criteria for genetic testing, unless they also have a family history of melanoma (96,100,101). Leachman and colleagues described a very useful rule to select patients for genetic testing in melanoma according to the melanoma incidence in the general population and the prevalence of mutations in each region. In countries with a low melanoma incidence, such

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as Southern European countries, the selection criteria for genetic counseling should follow the rule of two: individuals with two (synchronous or metachronous) primary melanomas and/or families with at least one invasive melanoma and one or more other diagnoses of melanoma and/or pancreatic cancers among first- or second-degree relatives on the same side of the family. Whilst countries with a moderate to high melanoma incidence, such as the USA and Northern European countries, should follow the rule of three: individuals with three or more primary invasive melanomas and families with at least one invasive melanoma and two more cases of melanoma and/or pancreatic cancer among first- or second-degree relatives on the same side of the family. For very high melanoma incidence countries, such as Australia, the rule of four may be suggested (102).

When genetic testing detects a melanoma predisposing mutation in a family, a screening cascade of individuals at risk is recommended. Individuals carrying *CDKN2A* mutations are included in prevention and early-detection programs that include the use of sun protection, dermatologic screening and self-skin examination, as an increased skin-cancer screening and surveillance by physicians and self-skin examination results in earlier detection of thinner melanomas (103-105). It has been demonstrated that melanoma genetic counseling has a positive impact on the improvement on total body skin examination and self-skin examination in unaffected individuals carrying germline mutations, after test reporting, while affected carriers maintain high levels of screening adherence (106). Furthermore, after melanoma genetic counseling unaffected members of high risk melanoma families report improvements in daily routine sun protection, showing that genetic counseling may motivate sustained improvements in prevention behaviors (107). Thus it is very important for both melanoma patients and unaffected individuals to be included in genetic counseling programs. Since an increased presence of smoking-related cancers has been detected in *CDKN2A* mutated families, advice to avoid smoking should also be included in the prevention programs (27,48). To date, individuals carrying *CDKN2A* mutations should be aware of the current lack of effective screening guidelines for pancreatic cancer (108), although magnetic resonance imaging surveillance can detect early-stage resectable pancreatic tumors (109).

In families where no *CDKN2A* mutation is identified, it should be stressed that the family is still at increased risk of melanoma on the basis of the family history. No further information on cancer risk is obtained, so these families should be managed according to family history (96). More studies should be necessary to understand the role of the other high-risk genes described to date (*BAP1*, *POT1*, *ACD*, *TERF2IP*, *TERT*, *CXCL1*), which methodology is better to asses them, or the role of the combination of the presence of different low to moderate risk variants, in *CDKN2A* wild-type families. The inclusion criteria for genetic testing of some of these genes also may differ. For example, *BAP1* genetic testing should be offered to families with a broader tumor spectrum including cutaneous and uveal melanoma, mesothelioma, RCC, and also with other skin tumors such as atypical intradermal tumors or basal cell carcinomas. The prevention and early detection programs for patients with mutations in *BAP1* should be modified based on the information provided of this cancer syndrome. *BAP1* mutation carriers should be closely monitored with, as an example, bi-annual dermatological and annual ophthalmological examinations and pulmonary/renal evaluations by imaging techniques (110). Moreover, many wild-type families for the known genes should carry private mutations that should be assessed by new approaches such as next generation sequencing, in order to detect the mutation responsible for the inherited susceptibility in the family and then improve the genetic counseling specifically in that family.

Conclusions

The new improvements in technology have led to the identification of new genes involved in melanoma susceptibility. However, the knowledge on melanoma susceptibility to date allows us to explain less than 30% of the genetic susceptibility in melanoma-prone families. In some cases the genetic susceptibility may be explained by the accumulation of the presence of low to moderate risk variants in the affected individuals. It is also possible that family aggregation can be explained by shared environmental exposures or also by chance. However next generation techniques can allow the identification of new genes involved in melanoma susceptibility.

Melanoma genetic counseling is important for patients and relatives to better understand the meaning of the disease and the genetic susceptibility. New studies are needed to assess the inclusion in genetic testing of the new genes related to melanoma susceptibility. However, knowing the genetic test results can also be important to refine the genetic counseling in the family as some of the

genes involved in melanoma susceptibility also predispose to other tumors. For this reason it is also important to analyze the role that the new genes identified in melanoma may play in the risk to develop other cancers.

Acknowledgements

Funding: The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants 09/01393 and 12/00840; CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain; AGAUR 2009 SGR 1337 and AGAUR 2014 SGR 603 of the Catalan Government, Spain; European Commission under the 6th Framework Programme, Contract No.LSHC-CT-2006-018702 (GenoMEL) and by the European Commission under the 7th Framework Programme, Diagnoptics; The National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115) and a grant from "Fundació La Marató de TV3, 201331-30", Catalonia, Spain. The work was carried out at the Esther Koplowitz Center, Barcelona. Miriam Potrony is the recipient of a PhD Fellowship (PFIS) from Instituto de Salud Carlos III, Spain.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Potrony M, Badenas C, Aguilera P, Puig-Butille JA, Carrera C, Malvehy J, Puig S. Update in genetic susceptibility in melanoma. Ann Transl Med 2015;3(15):210. doi: 10.3978/j.issn.2305-5839.2015.08.11

Annex 2

Additional Article 2

Title: Characterization of individuals at high-risk of developing melanoma in Latin America: bases for genetic counseling in melanoma.

Authors: Puig S, Potrony M, Cuellar F, Puig-Butille JA, Carrera C, Aguilera P, Nagore E, Garcia-Casado Z, Requena C, Kumar R, Landman G, Costa Soares de Sá B, Gargantini Rezze G, Facure L, de Avila AL, Achatz MI, Carraro DM, Duprat Neto JP, Grazziotin TC, Bonamigo RR, Rey MC, Balestrini C, Morales E, Molgo M, Bakos RM, Ashton-Prolla P, Giugliani R, Larre Borges A, Barquet V, Pérez J, Martínez M, Cabo H, Cohen Sabban E, Latorre C, Carlos-Ortega B, Salas-Alanis JC, Gonzalez R, Olazaran Z, Malvehy J, Badenas C.

Publication: Genet Med. 2016;18(7):727-36. Doi: 10.1038/gim.2015.160.

Impact Factor (JCR Science Edition 2016): 8.229 (1^{st} decile, GENETICS & HEREDITY)

Contribution of the doctoral student to the article: The doctoral student participated in the data joining from each center, performed the statistical data analysis and was responsible for writing the majority of the original draft of the manuscript.

Official journal of the American College of Medical Genetics and Genomics **ORIGINAL RESEARCH ARTICLE** Genetics inMedicine

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Characterization of individuals at high risk of developing melanoma in Latin America: bases for genetic counseling in melanoma

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Purpose: *CDKN2A* is the main high-risk melanoma-susceptibility gene, but it has been poorly assessed in Latin America. We sought to analyze *CDKN2A* and *MC1R* in patients from Latin America with familial and sporadic multiple primary melanoma (SMP) and compare the data with those for patients from Spain to establish bases for melanoma genetic counseling in Latin America.

Methods: *CDKN2A* and *MC1R* were sequenced in 186 Latin American patients from Argentina, Brazil, Chile, Mexico, and Uruguay, and in 904 Spanish patients. Clinical and phenotypic data were obtained.

Results: Overall, 24 and 14% of melanoma-prone families in Latin America and Spain, respectively, had mutations in *CDKN2A*. Latin American families had *CDKN2A* mutations more frequently ($P = 0.014$) than Spanish ones. Of patients with SMP, 10% of those from Latin America and 8.5% of those from Spain had mutations in *CDKN2A* ($P = 0.623$). The most recurrent *CDKN2A* mutations were c.-34G>T and p.G101W. Latin American patients had fairer hair ($P = 0.016$) and skin ($P < 0.001$) and a higher prevalence of *MC1R* variants ($P = 0.003$) compared with Spanish patients.

Conclusion: The inclusion criteria for genetic counseling of melanoma in Latin America may be the same criteria used in Spain, as suggested in areas with low to medium incidence, SMP with at least two melanomas, or families with at least two cases among first- or second-degree relatives.

Genet Med advance online publication 17 December 2015

Key Words: *CDKN2A*; familial; Latin America; melanoma; *MC1R*

INTRODUCTION

Melanoma is the most aggressive of common skin cancers because of its tendency to metastasize. Its incidence is rapidly increasing, especially among Caucasian populations. Melanoma is the second most diagnosed cancer among patients younger than 30 years of age,[1] and the 3-year survival rate for patients

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Submitted 13 July 2015; accepted 29 September 2015; advance online publication 17 December 2015. doi:10.1038/gim.2015.160

with metastases is around 15%.[2] Identification of individuals at high risk of developing melanoma is necessary since an early diagnosis improves the disease prognosis.[3]

Melanoma is caused by the interaction of environmental, phenotypic, and genetic factors. The main environmental risk factor for melanoma is sun exposure.[4] Individuals with fair skin, red hair, and/or a high nevi count have an increased risk of developing melanoma.[5] To date, *CDKN2A*, which encodes the tumor suppressor proteins p16INK4A and p14ARF, is the major high-risk gene involved in melanoma susceptibility.[6] *CDKN2A* has been widely studied in melanoma patients from the United States, Europe, and Australia.[6] The frequency of germline mutations in *CDKN2A* varies across populations (5–72%) and depends on the selection criteria used.[6,7] Haplotype analysis indicates a founder effect for most of the recurrent mutations detected.[8] Identification of the prevalence of *CDKN2A* mutations in patients at high risk for melanoma and the correlation of these mutations with clinical data has been crucial for establishing genetic counseling for melanoma. Melanoma risk may also be modulated by common genetic variants acting as low- to medium-penetrance variants.[9] *MC1R* plays a key role in pigmentation and is responsible for phenotypic characteristics such as hair and skin color and the capacity of response to ultraviolet radiation.[10] Several *MC1R* variants are associated with a moderately increased melanoma risk and also modulate the effect of *CDKN2A* mutations in carriers.[11]

Genetic counseling and specific dermatological follow-up may be offered to patients at high risk for melanoma.[12] In countries with a low to medium incidence of melanoma, genetic counseling is offered to patients with two primary melanomas and/or to families with two melanoma cases and/or one pancreatic adenocarcinoma and one melanoma in first- or second-degree relatives (the "rule of two"). In countries with a moderate to high incidence of melanoma, however, genetic counseling is offered to patients with three primary melanomas and to families with three cases of melanoma or pancreatic cancer in first- or second-degree relatives (the "rule of three").[13] It has been demonstrated that melanoma genetic counseling has a positive impact on the improvement of total body skin examination and self-examination of the skin in unaffected individuals carrying germline mutations after test reporting, whereas affected carriers maintain high levels of screening adherence.[14] Furthermore, after melanoma genetic counseling, unaffected members of high-risk melanoma families report improvements in daily routine sun protection, showing that genetic counseling may motivate sustained improvements in prevention behaviors.[15] Thus it is very important for both melanoma patients and unaffected individuals from the family to be included in genetic counseling programs.

Few studies have assessed the prevalence of *CDKN2A* mutations or *MC1R* variants and phenotypic characteristics in patients at high risk for melanoma from Latin American countries. *CDKN2A* mutations have been identified in 13.6% of melanoma-prone families from São Paulo, Brazil,[16] whereas one study reported no mutations in Porto Alegre,[17] and in a different cohort the mutation frequency was 7%.[18] In melanoma-prone families from Uruguay, 5/6 families had *CDKN2A* mutations.[19] Phenotypic and genetic characterization of individuals at high risk for melanoma from Latin America may improve their management and implement genetic counseling in these countries. We present the molecular characterization of *CDKN2A* and *MC1R* genes in the largest set of patients at high risk for melanoma from distinct Latin American countries (Argentina, Brazil, Chile, Mexico, and Uruguay), and we compare the data with two sets of Spanish patients at high risk for melanoma to establish bases for genetic counseling in Latin America.

MATERIALS AND METHODS

The multicenter cross-sectional study included 1,090 patients at high risk for melanoma: 758 patients with familial melanoma (FM) and 332 patients with SMP from Latin American countries and Spain. Because Latin America is a region with a low incidence of melanoma (GLOBOCAN 2012, World Health Organization; http://globocan.iarc.fr), the inclusion criteria followed the rule of two.

Overall, 186 Latin American melanoma patients were recruited from Argentina (n = 10), Chile (n = 28), Mexico (n = 6), Uruguay (n = 25), and Brazil (n = 117), which included two sets of patients: Porto Alegre (Southern Brazil) (n = 58) and São Paulo (southeast region) (n = 59). The contribution of each country to the study resulted in a broad representation of a number of Latin American countries. A set of 904 Spanish patients with melanoma from Barcelona (n = 706) and Valencia (n = 198) also were included using the same selection criteria.

The number of primary melanomas, age at diagnosis, number of melanoma cases in the family, ancestral origin, and phenotypic data (hair and eye color, skin phototype, and nevi count) were recorded by dermatologists for most of the patients. Although the number of missing values was higher in the set of Spanish patients than in the Latin American patients, this did not introduce a bias, and the information recruited was informative for the whole cohort: Spanish patients were recruited consecutively, and missing data were distributed randomly; two different cohorts from Spain where used to minimize the bias due to the data collection procedure; and the variable with the greatest amount of missing data had information from at least 600 Spanish patients. Partial genetic information of the patients with melanoma from Spain and Brazil, and a subset of pedigrees from Uruguay, has been previously reported.[16–21]

The study was approved by the ethical committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

CDKN2A and *MC1R* molecular screening

Molecular characterization of *CDKN2A* was performed in all patients. *CDKN2A* was sequenced in all patients, as previously described.[16,18,20,21] *MC1R* was sequenced as described elsewhere.[22,23] The *MC1R* genotype was available from all patients from Argentina and Chile, 57% (33/58) patients from Porto Alegre, Brazil, 92% (54/59) patients from São Paulo, Brazil, 96% (24/25)

patients from Uruguay, 59% (419/706) patients from Barcelona, Spain, and 94% (186/198) patients from Valencia, Spain. *MC1R* genotype data were not available for patients from Mexico.

Statistical analyses

For the statistical analyses, the most common *MC1R* variants were classified as r variants (not associated with red hair color: p.V60L, p.V92M, p.R163Q) or R variants (associated with red hair color: p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H).[10]

SPSS software version 17.0 (IBM, Chicago, IL) was used. Two-sided Pearson χ^2 or Fisher exact tests were used for categorical variables, as applicable. Student's *t*-test was used for quantitative variables. Adjusted *P* values were calculated using the Bonferroni correction. The test was considered significant if the *P* value or adjusted *P* value (as applicable) was <0.05.

RESULTS

The study included a set of 1,090 patients with melanoma from distinct Latin American countries and Spain. Latin America and Spain had similar frequencies of FM cases (67.7 and 69.9%, respectively) and SMP (32.3 and 30.1%, respectively; $P = 0.600$), and there were no gender differences (40.3% male and 58.7% female vs. 41.5% male and 58.5% female, respectively; $P = 0.806$). Since Latin America is a mixed population from European, Native, African and Asian origin as a result of the colonization process and migratory effects,[24] we collected information regarding the patients' ancestral origin. The four grandparents of more than 70% of Latin American patients were of European origin. Latin American and Spanish patients differed in pigmentation traits. Latin American patients had fairer hair color (adjusted $P = 0.016$) and skin phototype (adjusted $P < 0.001$) than Spanish patients. No differences were observed for nevi count or eye color (**Table 1**).

Considering all patients, *CDKN2A* mutation prevalence was 19% in Latin America and 12% in Spain. *CDKN2A* mutation frequency in SMP was similar in Latin America (10%) and Spain (8.5%) ($P = 0.623$). However, the prevalence of *CDKN2A* mutations in Latin American melanoma-prone families was higher than in Spain (24 and 14%, respectively; $P = 0.019$). The frequency of mutations varied among countries. Whereas southern Brazil had a low mutation prevalence, Chile and Uruguay showed a high prevalence of mutations in both SMP and FM (**Table 2**).

The *CDKN2A* mutations differed in each country (**Table 3**). Overall, 74% (23/31) of Latin American *CDKN2A* mutation carriers had a mutation also found in Spanish patients with melanoma. The most prevalent mutations in Latin America (c.-34G>T and p.G101W (c.301G>T)) were among the most recurrent mutations in Spain, which are p.G101W (33%), p.V59G (c.176T>G) (7%), c.-34G>T (6%), p.A36RfsX17 (c.106delG) (6%), and p.E120fsX145 (c.358delG) (5%) (**Table 3**). Mutation c.-34G>T was present in 90% of families from Chile, and families from São Paulo (Brazil) and Uruguay with *CDKN2A* mutations. Mutation p.G101W was present in families from Argentina, São Paulo (Brazil), and Uruguay. The other mutations detected in Latin America were restricted to a few pedigrees.

CDKN2A mutations have been previously associated with a lower age at diagnosis, number of primary melanomas, and the number of cases in the family.[6] The whole set of patients also showed these associations (**Table 4**). Latin American patients with melanoma carrying a *CDKN2A* mutation had an increased number of cases in the family and a lower age at diagnosis, but the number of personal primary melanomas did not reach significance.

We sequenced *MC1R* to assess the distribution of *MC1R* variants across countries (**Table 5**). We observed differences in the number and type of variants between Latin America and Spain. We detected *MC1R* variants in 80.5% of Latin American and 67.9% of Spanish patients ($P = 0.003$), with a similar R variant frequency (39.6 vs. 36.3%, respectively; $P = 0.514$) but a higher r variant prevalence in Latin America (40.9 vs. 31.6%, respectively; $P = 0.033$). We analyzed the frequencies of the most common R and r variants, comparing Latin America and Spain (**Supplementary Table S1** online). When adjusting using the Bonferroni correction, we found a significantly increased presence of p.R160W (17.4 vs. 7.5%; adjusted $P < 0.005$) and p.R163Q (14.1 vs. 5.2%; adjusted $P < 0.005$) in Latin America, but we should take into consideration that all patients carrying the p.R163Q variant in this study were from only three study sites: Brazil (São Paulo), Chile, or Uruguay. The p.D294H variant was more frequent in Spain (5.4 vs. 13.3%; adjusted $P = 0.045$). The presence of *MC1R* variants and R variants correlated with phenotype (**Supplementary Tables S2 and S3** online).

DISCUSSION

Latin America has a low incidence of melanoma (GLOBOCAN 2012). The characterization of melanoma genes has allowed other areas with low to medium incidence of melanoma, such as Spain, to recommend genetic counseling for patients with melanoma.[12,25] To date, only a few specialized centers in Latin America offer melanoma genetic counseling, and there is little knowledge of the implication of high-risk genes in melanoma susceptibility. This study presents the clinical and molecular characterization of *CDKN2A* and *MC1R* in the largest set of Latin American patients at high risk for melanoma.

CDKN2A mutation frequency in melanoma-prone families was higher in Latin America than Spain, using the same selection criteria. By contrast, both areas had similar SMP *CDKN2A* mutation prevalence, consistent with that reported in other studies (8.2–9%).[25,26] The age at diagnosis and number of primary melanomas were associated with the presence of mutations in *CDKN2A*, as previously reported.[6] Otherwise, we did not find associations between *CDKN2A* mutation and nevi count, suggesting that other genes could play a role in nevogenesis.[27,28] Most *CDKN2A* mutations identified had been previously detected in European or North American patients with melanoma. The most prevalent mutation in Latin America was c.-34G>T. This mutation occurs at a high

Table 1 Characteristics and phenotypic data of patients with melanoma, by country (region)

	Argentina		Brazil (Porto Alegre)		Brazil (São Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		Latin America		Spain		Adjusted	Total	
	n	%	*n*	%	*N*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*P* value[a]	*N*	%
Patients with melanoma																							
FM[b]	5	50.0	45	77.6	37	62.7	16	57.1	3	50.0	20	80.0	472	66.9	160	80.8	126	67.7	632	69.9	—	758	69.5
SMP	5	50.0	13	22.4	22	37.3	12	42.9	3	50.0	5	20.0	234	33.1	38	19.2	60	32.3	272	30.1		332	30.5
Total	10		58		59		28		6		25		706		198		186		904			1,090	
Phenotypic characteristics																							
Hair color																							
Red	1	10.0	5	14.7	2	3.4	1	3.6	1	16.7	4	19.0	30	5.8	16	9.4	14	8.9	46	6.7	**0.016**	60	7.1
Blond	2	20.0	17	50.0	26	44.1	8	28.6	1	16.7	2	9.5	126	24.4	37	21.8	56	35.4	163	23.7		219	25.9
Dark	7	70.0	12	35.3	31	52.5	19	67.8	4	66.7	15	71.4	361	69.8	117	68.8	88	55.7	478	69.6		566	67.0
Missing	0		24		0		0		0		4		189		28		28		217			245	
Total	10		58		59		28		6		25		706		198		186		904			1,090	
Eye color																							
Fair	5	50.0	20	58.8	27	45.8	14	50.0	2	33.3	9	42.9	226	43.9	52	29.9	77	48.7	278	40.3	0.240	355	41.9
Dark	5	50.0	14	41.2	32	54.2	14	50.0	4	66.7	12	57.1	289	56.1	122	70.1	81	51.3	411	59.7		492	58.1
Missing	0		24		0		0		0		4		191		24		28		215			243	
Total	10		58		59		28		6		25		706		198		186		904			1,090	
Skin color[c]																							
Fair	7	70.0	56	98.2	51	86.4	21	75.0	3	50.0	18	85.7	299	55.2	75	42.1	156	86.2	374	51.9	**<0.001**	530	58.8
Dark	3	30.0	1	1.8	8	13.6	7	25.0	3	50.0	3	14.3	243	44.8	103	57.9	25	13.8	346	48.1		371	41.2
Missing	0		1		0		0		0		4		164		20		5		184			189	
Total	10		58		59		28		6		25		706		198		186		904			1,090	
Nevi count																							
<50	—		11	47.8	38	64.4	9	32.1	4	66.7	12	63.2	195	38.7	72	73.5	74	54.8	267	44.4	0.332	341	46.3
50—100	—		3	13.0	10	16.9	9	32.1	1	16.7	3	15.8	130	25.8	21	21.4	26	19.3	151	25.1		177	24.0
>100	—		9	39.1	11	18.6	10	35.7	1	16.7	4	21.1	179	35.5	5	5.1	35	24.9	184	30.6		219	29.7
Missing	10		35		0		0		0		6		202		100		51		302			353	
Total	10		58		59		28		6		25		706		198		186		904			1,090	
Ancestral origin[d]																							
European	8	88.9	44	78.6	35	59.3	20	74.1	4	66.6	20	80.0	—	—	—	—	131	73.2	—	—	—	—	—
Latin	1	11.1	12	21.4	20	33.9	5	18.5	1	16.7	1	20.0	—	—	—	—	40	22.3	—	—		—	—
Amerindian	0	0	0	0	0	0	2	7.4	1	16.7	0	0	—	—	—	—	3	1.7	—	—		—	—
African American	0	0	0	0	4	6.8	0	0	0	0	1	20.0	—	—	—	—	5	2.8	—	—		—	—
Missing	1		2		0		1		0		3		—	—	—	—	7		—	—		—	—
Total	10		58		59		28		6		25		—	—	—	—	186		—	—		—	—

Statistically significant *P* values are given in bold.
SMP, sporadic multiple primary melanoma.
[a]*P* values were obtained comparing Latin America with Spain. Bonferroni correction was used to obtain the adjusted *P* values. [b]In the familial melanoma (FM) category we included families with at least two melanoma cases.. [c]Skin color was classified according to the Fitzpatrick phototype classification: fair (phototypes I or II) and dark (phototypes III–V). [d]The ancestral origin of the Latin American patients with melanoma is indicated according to the origin of their grandparents. "European" means all grandparents were born in a European country. "Latin," "Amerindian," and "African American" indicate that at least one of the grandparents was born in Latin America (but had Spanish or Portuguese ancestors), was a descendent of natives, or had an African-American origin, respectively. In the case that the grandparents were of different origins, for example, African American and Amerindian, the ancestral origin was indicated taking into consideration the darkest skin color: African American/Amerindian/Latin. There were no individuals with Asian origin.

Table 2 *CDKN2A* mutation distribution between families according to the number of melanoma cases by country (region)

Country (region)	All pedigrees included (*N*)	Patients with SMP					All melanoma-prone families					Families with two cases					Families with three cases					Families with four or more cases				
		Total		*CDKN2A* mutation		*P* value	Total		*CDKN2A* mutation		*P* value	Total		*CDKN2A* mutation			Total		*CDKN2A* mutation			Total		*CDKN2A* mutation		
		n	%	*n*	%		*n*	%	*n*	%		*n*	%	*n*	%		*n*	%	*n*	%		*n*	%	*n*	%	
Argentina	10	5	50.0	1	20.0		5	50.0	0	0		4	80.0	0	0		1	20.0	0	0		0	0	0	0	
Brazil (Porto Alegre)	50	13	26.0	0	0		37	74.0	3	8.1		27	73.0	1	3.7		7	18.9	1	14.3		3	8.1	1	33.3	
Brazil (São Paulo)	57	22	38.6	1	3.6		35	61.4	8	22.4		21	60.0	1	4.8		13	37.1	7	53.8		1	2.9	0	0	
Chile	27	12	44.4	3	25.0		15	55.6	7	46.7		12	80.0	5	41.7		1	6.7	0	0		2	13.3	2	100	
Mexico	5	3	60.0	0	0		2	40.0	1	50.0		2	100	1	50.0		0	0	0	0		0	0	0	0	
Uruguay	20	5	25.0	1	20.0		15	75.0	8	40.0		11	73.3	4	36.4		4	26.7	3	75.0		0	0	0	0	
Spain (Barcelona)	564	234	41.5	20	8.5		330	58.5	47	14.2		269	81.5	30	11.2		47	14.2	11	23.4		14	4.2	6	42.9	
Spain (Valencia)	147	38	25.9	3	7.9		109	74.1	15	13.8		79	72.5	8	10.1		22	20.2	5	22.7		8	7.3	2	25.0	
Missing	0	0	—	0	—		0	—	0	—		0	—	0	—		0	—	0	—		0	—	0	—	
Latin America	169	60	35.5	6	10.0	0.623	109	64.5	26	23.9	**0.019**	77	70.6	12	15.6		26	23.9	11	42.3		6	5.5	3	50.0	**0.007**
Spain	711	272	38.3	23	8.5		439	61.7	62	14.1		348	79.3	38	10.9		69	15.7	16	23.2		22	5.0	8	36.4	**<0.001**
Total	880	332	37.7	29	8.9		548	62.3	88	16.1		425	77.6	50	11.8		95	17.3	27	28.4		28	5.1	11	39.3	**<0.001**

Statistically significant *P* values are given in bold.

[a]*P* values to assess differences in mutation frequency among families with sporadic multiple primary melanoma (SPM) and among all melanoma-prone families were obtained by comparing the global result of Latin America versus Spain. [b]*P* values to assess differences in mutation frequency among families according to the number of melanoma cases were assessed separately in Latin America, in Spain, and in the entire set of patients (total).

Table 3 *CDKN2A* genetic results

Exon	Protein change		Argentina		Brazil (Porto Alegre)		Brazil (São Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		Latin America		Spain		Total	
cDNA change	p14ARF	p16INK4A	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*N*	%
1β																								
c.127G>C	p.V43L	—	0	0	0	0	1	11.1	0	0	0	0	0	0	0	0	0	0	1	3.4	0	0	1	0.9
1α																								
c.-34G>T	—	—	0	0	1	33.3	3	33.3	9	90	0	0	1	12.5	5	7.5	0	0	14	45.2	5	5.9	19	16.2
c.106delG	—	p.A36RfsX17	0	0	0	0	0	0	0	0	0	0	0	0	5	7.5	0	0	0	0	5	5.9	5	4.2
c.142C>A	—	p.P48T	0	0	1	33.3	3	33.3	0	0	0	0	0	0	0	0	0	0	4	12.9	0	0	4	3.4
c.146T>C	—	p.I49T	0	0	0	0	0	0	0	0	1	100	0	0	0	0	0	0	1	3.2	0	0	1	0.9
2																								
c.159G>C	p.D68H	p.M53I	0	0	1	33.3	0	0	0	0	0	0	0	0	0	0	0	0	1	3.2	0	0	1	0.9
c.176T>G	p.S73R	p.V59G	0	0	0	0	0	0	0	0	0	0	0	0	2	3.0	4	22.2	0	0	6	7.0	6	5.1
c.262G>T	p.G102V	p.E88X	0	0	0	0	0	0	0	0	0	0	2	25.0	2	3.0	0	0	2	6.5	2	2.4	4	3.4
c.301G>T	p.R115L	p.G101W	1	100	0	0	1	11.1	0	0	0	0	5	62.5	24	35.8	4	22.2	7	22.6	28	32.9	35	29.9
c.358delG	—	p.E120DfsX145	0	0	0	0	0	0	0	0	0	0	0	0	1	1.5	3	16.7	0	0	4	4.7	4	3.4
c.430C>T	—	p.R144C	0	0	0	0	0	0	1	10	0	0	0	0	0	0	0	0	1	3.2	0	0	1	0.9
3																								
IVS2-105A>G	—	—	0	0	0	0	1	11.1	0	0	0	0	0	0	0	0	0	0	1	3.2	0	0	1	0.9
All exons	Other[a]	Other[a]	0	0	0	0	0	0	0	0	0	0	0	0	28	41.7	7	38.9	0	0	35	41.2	35	29.9
Total			1		3		9		10		1		8		67		18		31		85		117	
	p16INK4A Polymorphism p.A148T																							
	Yes		—	—	7	12.1	5	8.6	1	4.0	—	—	3	12	65	9.2	15	7.6	16	9.6	80	8.9	96	9.0
	No		—	—	51	87.9	53	91.4	24	96.0	—	—	22	88	638	90.8	183	92.4	150	90.4	821	91.1	971	91.0
	Missing		10		0		1		3		6		0		3		0		20		3		23	
	Total		10		58		59		28		6		25		706		198		186		904		1,090	

There were no statistical differences between the prevalence of the p.A148T polymorphism among melanoma patients in Latin America and Spain ($P = 0.768$).

[a]The other *CDKN2A* mutations identified in the Spanish population affecting only p14ARF were p.R21RfsX46 (c.60ins16), p.G32R (c.94G>A), and p.A121T (c.318G>A); those affecting p16INK4A were p.A5T (c.13G>A), p.P11T (c.31C>A), p.G35E (c.104G>A), p.N39S (c.116A>G), p.Y44X (c.131dup), p.Q50R (c.149A>G), p.G55V (c.164G>T), p.L65P (c.194T>C), p.N71S (c.212A>G), p.R80X (c.238C>T), p.P81S (c.241C>T), p.D84Y (c.250G>T), p.R87W (c.259C>T), p.R99W (c.295C>T), p.G101R (c.301G>A), p.A102V (c.305C>T), p.R112P (c.335G>C), p.E120SfsX21 (c.359_365del), p.G122R (c.364G>C), p.A127S (c.379G>A), and p.D153N (c.457G>A).

Table 4 Clinical and phenotypic characteristics of melanoma patients according to the presence of a *CDKN2A* mutation, by country

	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		Latin America		Adj.	Spain		Adj. *P*	Total		Adj. *P*
	n	%	*n*	%	*N*	%	*N*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*P*	*N*	%		*N*	%	
CDKN2A mutation carriers																									
Hair color																									
Red	0	0	0	0	1	50.0	0	0	0	0	3	75.0	7	23.3	2	12.5	4	28.6	**0.025**	9	19.6	**0.015**	13	21.7	**<0.002**
Blond	0	0	0	0	2	7.7	3	37.5	0	0	0	0	9	7.1	2	5.4	5	8.9		11	6.7		16	7.3	
Dark	1	14.3	1	8.3	9	29.0	7	36.8	2	50.0	7	46.7	65	18.0	13	11.1	27	30.7		78	16.3		105		
Missing	0		2	—	0		0		0		1		20		2		3			22			25		
Total	1		3	—	12		10		2		11		101		19		39			120			159		
Eye color																									
Fair	0	0	1	5.0	5	18.5	4	28.6	0	0	4	44.4	31	13.7	6	11.5	14	18.2	0.950	37	13.3	1.000	51	14.4	1.000
Dark	1	20.0	0	0	7	21.9	6	42.9	2	50.0	6	50.0	49	17.0	11	9	22	27.2		60	14.6		82	16.7	
Missing	0		2		0		0		0		1		21		2		3			23			26		
Total	1		3		12		10		2		11		101		19		39			120			159		
Skin color[a]																									
Fair	1	14.3	3	5.4	11	21.6	9	42.9	1	33.3	10	55.6	46	15.4	4	5.3	35	22.4	1.000	50	13.4	1.000	85	16.0	1.000
Dark	0	0	0	0	1	12.5	1	14.3	1	33.3	0	0	35	14.4	15	14.6	3	12.0		50	14.5		53	14.3	
Missing	0		0		0		0		0		1		20		0		1			20			21		
Total	1		3		12		10		2		11		101		19		39			120			159		
Nevi count																									
<50	—	—	0	0	10	26.3	2	22.2	1	25.0	8	66.7	26	13.3	8	11.1	21	28.4	0.765	34	12.7	0.260	55	16.1	1.000
50—100	—	—	0	0	1	10.0	1	11.1	1	100	0	0	31	23.7	3	14.3	3	11.5		33	21.9		36	20.3	
>100	—	—	1	11.1	1	9.1	7	70.0	0	0	2	50.0	29	16.2	0	0	11	31.4		29	15.8		40	18.3	
Missing	—	—	2		0		0		0		1		16		8		4			24			28		
Total	—	—	3		12		10		2		11		102		19		39			120			159		
No. of MMs																									
1	0	0	1	3.7	4	16.7	5	35.7	1	100	1	100	43	12.3	13	9.5	12	16.7	1.000	56	11.5	**<0.002**	68	12.2	**<0.002**
2	1	25.0	1	5.9	5	22.7	4	36.4	0	0	3	42.9	33	12.3	3	6.1	14	22.2		36	11.4		50	13.2	
3	0	0	1	10.0	1	14.3	1	33.3	1	33.3	0	0	12	22.2	2	25	4	16.7		14	22.6		18	20.9	
≥4	0	0	0	0	2	33.3	0	0	0	0	3	100	12	48	1	25	5	38.5		13	44.8		18	42.9	
Missing	0		0		0		0		0		4		1		0		4			1			5		
Total	1		3		12		10		2		11		102		19		39			120			159		
No. of A148T carriers with melanoma																									
1	—	—	5	16.1	1	3.4	0	0	—	—	0	0	33	9.5	8	5.8	6	8.1	1.000	41	8.4	1.000	47	8.4	1.000
2	—	—	1	5.9	4	19.0	0	0	—	—	1	14.3	24	9.0	4	8.2	6	10.9		28	8.9		34	9.2	
≥3	—	—	2	14.3	0	0	1	33.3	—	—	0	0	7	9.0	3	25.0	3	9.1		10	11.1		13	10.6	
Missing	—	—	0		0		0		—	—	2		1		0		2			1			3		
Total	—	—	8		5		1		—	—	3		65		15		17			80			97		
Age at diagnosis of first melanoma[b]	Years	SD	Years	SD	Years	SD	Years	SD	Years	SD	Years	SD	Years	SD	Years	SD	Years	SD	*P* value	Years	SD	*P* value	Years	SD	*P* value
CDKN2A mutation	39.0	—	40.3	18.0	43.7	10.6	43.4	9.8	47.5	3.5	36.3	15.0	39.4	12.7	42.7	15.4	41.5	11.9	**0.006**	40.0	13.2	**<0.001**	40.4	12.8	**<0.001**
CDKN2A wild type	44.9	13.6	50.0	13.6	45.7	13.2	52.6	16.4	39.2	6.8	51.8	17.3	47.2	16.6	47.8	16.8	48.5	13.8		47.4	16.6		47.5	16.2	
Total	44.3	12.9	49.5	13.8	45.3	12.6	49.2	14.8	42.0	6.9	44.1	17.7	46.1	16.2	47.3	16.7	47.1	13.7		46.4	16.4		46.5	16.0	

P values were obtained by comparing carriers vs. noncarriers individually in Latin America, Spain, and the total sample. Adjusted *P* values were calculated using the Bonferroni correction. Statistically significant *P* values are given in bold.
MM, primary melanoma.
[a]Skin color was classified according to the Fitzpatrick phototype classification: fair (phototypes I or II) and dark (phototypes III–V). [b]The age at diagnosis of first melanoma was not available in 1/28 (3.4%) of Chilean, 2/20 (10%) of Uruguayan, 64/707 (9.1%) of Barcelonan, and 3/195 (1.5%) of Valencian patients.

Table 5 *MC1R* variant distribution

	Argentina		Brazil (Porto Alegre)		Brazil (São Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		Latin America		Spain		*P* value	Total	
	n	%	*n*	%	*N*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%		*N*	%
MC1R WT	1	10.0	10	30.3	9	16.7	6	21.4	—	—	3	12.5	118	28.2	75	41.2	29	19.5	193	32.1	**0.003**	222	29.6
≥1 *MC1R* variant[a]	9	90.0	23	69.7	45	83.3	22	78.6	—	—	21	87.5	301	71.8	107	58.8	120	80.5	408	67.9		528	70.4
R/R, R/r, or R/WT	4	40.0	10	30.3	27	50.0	7	25.0	—	—	11	45.8	168	40.1	50	27.5	59	39.6	218	36.3	0.507	277	36.9
r/r or rWT	5	50.0	13	46.4	18	33.3	15	53.6	—	—	10	41.7	133	31.7	57	31.3	61	40.9	190	31.6	**0.033**	253	33.4
Missing	0		25		5		0		6		1		287		12		37		303			340	
Total	10		58		59		28		6		25		706		198		186		904			1,090	

P values were obtained comparing Latin America versus Spain. Statistically significant *P* values are given in bold.

R, *MC1R* variant associated with the red hair color phenotype (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H, and rare frameshift variants); r, *MC1R* variants not associated with the red hair color phenotype (p.V60L, p.V92M, p.R163Q, and other rare missense variants); WT, wild type. [a]Synonymous variants were considered *MC1R* WT; all other missense or frameshift nucleotide changes, either prevalent or rare, were considered *MC1R* variants.

frequency among unrelated families from Chile, suggesting a possible founder effect. In one family from Chile we detected p.R144C (c.430C>T), previously detected at the germline level in a patient with pancreatic cancer.[29] Mutation p.G101W is also frequent in Latin America, as in Mediterranean countries (Italy, France, and Spain)[7] where haplotype analysis showed a founder effect.[30] We identified four other mutations in Brazil: p.P48T (c.142C>A), previously reported in an Italian population with FM,[31] was found in four families, one of them of Italian ancestry, suggesting a possible founder effect[32]; IVS2-105A>G and p.M53I (c.159G>C), previously reported in melanoma-prone families from the United Kingdom, Australia, and the United States[7]; and mutation p.V43L (c.127G>C), affecting p14ARF, which has not previously been reported. In Uruguay we detected p.E88X (c.262G>T) in two families, which also was detected in two Spanish pedigrees. In Mexico we identified a mutation in the two probands of one family—p.I49T (c.146T>C)—which was previously reported in a case of FM by Hussussian et al.[33] and did not segregate with melanoma in that case. However, functional analysis showed impairment for this variant.[34]

We detected differences in *MC1R* variant distribution in our set of patients. Latin American patients with melanoma carry more *MC1R* variants. These genetic results correlate with the phenotypic data, where Latin American patients with melanoma have fairer skin and hair color. The prevalence of *MC1R* variants varies between populations.[35] In this study, specific variant frequencies differed between Latin American and Spanish patients with melanoma. Latin American patients with melanoma had an increased presence of p.R160W and p.R163Q. However, controls would be needed to assess the melanoma risk associated with carrying these variants in Latin America. p.R160W is associated with an increased risk for melanoma and red hair color.[10] By contrast, p.R163Q, which is not associated with pigmentation or tanning response, favors the development of chronic sun exposure melanomas in the Mediterranean population[22] and increases the risk for melanoma in areas with high ultraviolet radiation.[36] These reports suggest that a possible interaction between p.R163Q and a high ultraviolet radiation dose could favor melanoma development. Most Latin American countries receive a huge amount of ultraviolet radiation compared with northern latitudes; this could explain the increased frequency of SMP and FM with the p.R163Q variant in Latin America, although its frequency in a control Latin American population is unknown.

To date, genetic testing in patients at high risk for melanoma is restricted to *CDKN2A* and *CDK4*. More studies of patients wild type for these genes should be conducted to assess the role of other melanoma-susceptibility genes such as *MITF*, *BAP1*, *TERT*, *POT1*, *ACD*, and *TERF2IP*[8] for their possible incorporation in melanoma genetic counseling. In this study we demonstrated that *CDKN2A* germline mutation frequency in melanoma-prone families with at least two melanoma cases is greater in Latin America than Spain (23.9 vs. 14.1%, respectively). Inclusion criteria for genetic testing of melanoma in Spain follow the rule of two.[12] Based on the results of this

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study, the inclusion criteria for genetic counseling for patients with melanoma in Latin America should also follow this rule because it allows the detection of *CDKN2A* mutations in a significant number of patients, except for southern Brazil, where the rule of three should be used. Genetic testing allows us to identify mutation carriers in families with a high risk of developing the disease. Carriers can be included in specific follow-up programs that allow the detection of melanomas at early stages, which improves the disease prognosis.[3,37,38] Digital follow-up with specific dermatologic techniques, including total-body photography and digital dermoscopy, allow early detection of melanomas with a low rate of excision.[38] Early melanomas in patients carrying *MC1R* variants may be difficult to diagnose definitively using dermoscopy, and an integrated approach including clinical history and dermoscopic data should be used when evaluating them.[39] Thus, *MC1R* sequencing could also help to choose the best screening methods. The experience of genetic counseling in Spain over 10 years shows that melanomas can be diagnosed at any time, so the follow-up of individuals at high risk for melanoma should be maintained over time.[12]

In conclusion, Latin American patients with melanoma and at high risk for melanoma had fair skin and European origin. The mutations found also had been detected in Spanish, European, or North American populations, suggesting that they could have a single origin and that there could be a founder effect. Finally, inclusion criteria for genetic counseling in Latin American patients with melanoma should follow the rule of two: two primary melanomas in an individual or families with at least one invasive melanoma and one or more other diagnoses of melanoma or pancreatic cancer in first- or second-degree relatives.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

The main funding for the study was provided by GenoMEL (contract LSHC-CT-2006–018702) and by the National Cancer Institute of the US National Institutes of Health (CA83115). The research at the Melanoma Unit in Barcelona is partially funded by grants 03/0019, 05/0302, 06/0265, 09/1393, and 12/00840 from Fondo de Investigaciones Sanitarias, Spain; by the *CIBER de Enfermedades Raras* of the Instituto de Salud Carlos III, Spain; by AGAUR 2014_SGR_603 of the Catalan Government, Spain; and by the European Commission under the 6th Framework Programme. M.P. is the recipient of a PhD Fellowship (PFIS) from Instituto de Salud Carlos III, Spain. F.C. was partially funded by a scholarship (152256/158706) from Consejo Nacional de Ciencia y Tecnología (CONACYT), México. The research at São Paulo, Brazil, was funded by Fundação para o Amparo da Pesquisa do Estado de São Paulo (FAPESP), São Paulo, Brazil (2007/04313-2). The research at Porto Alegre city, Brazil, was funded by the Brazilian Post-Graduation Agency Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The research in Uruguay was funded by Comisión Honoraria de Lucha Contra el Càncer and Fundación Manuel Pérez, Montevideo, Uruguay. The authors give special thanks to the coordinator of the RO1 grant, David Elder, and those of the FP6 grant, Julia Newton-Bishop and Nelleke Gruis, for their support in the development of the project. The authors also thank their patients and their families, who are the main reason for our studies; the nurses from the Melanoma Unit of Hospital Clínic of Barcelona; Daniel Gabriel, Pablo Iglesias, and Maria E. Moliner for helping to collect patient data; Amanda de Nobrega from São Paulo; Thomas Ruzicka, Carola Berking, and the Department of Dermatology and Allergology of Ludwig Maximilian University, Munich, Germany, for support in performing p.A148T analyses in Brazilian cases from HCPA and Carolina Ribas do Nascimento for performing *MC1R* tests in HCPA; Lídice Dufrechou from Uruguay and Helena Kruyer for helping with English editing and correction of the manuscript. In addition, the authors thank Victoria Godinez Puig from Mexico for helping to collect patient samples and data, as well as the Biobanco del Instituto Valenciano de Oncología and the A.C. Camargo Biobank.

Statement on prior presentation: part of the results of this study were presented as an Oral Communication in the GenoMEL/BioGenoMEL annual meeting 2014, Valencia, Spain, 7–9 May 2014.

Role of the sponsors: the sponsors had no role in the design and conduct of the study; in the collection, analysis, and interpretation of data; or in the preparation, review, or approval of the manuscript.

DISCLOSURE

The authors declare no conflict of interest.

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Table S1. *MC1R* specific common variants frequency across the different Latin American countries and Spain

	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		**LATIN AMERICA**		**SPAIN**			**TOTAL**	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	Adj. *P*	*N*	%
≥1 p.V60L	3	30	13	39.4	23	42.6	5	17.9	-	-	6	25	105	25.1	51	28	50	33.6	156	26	0.594	206	27.5
≥1 p.D84E[a]	0	0	1	3.0	2	3.7	0	0	-	-	0	0	2	0.5	0	0	3	2.0	2	0.3	0.504	5	0.7
≥1 p.V92M	1	10	3	9.1	4	7.4	1	3.6	-	-	5	20.8	48	11.5	19	10.4	14	9.4	67	11.1	1.000	81	10.8
≥1 p.R142H[a]	1	10	0	0	0	0	1	3.6	-	-	3	12.5	16	3.8	9	4.9	5	3.4	25	4.2	1.000	30	4.0
≥1 p.R151C[a]	2	10	6	18.2	8	14.8	1	3.6	-	-	2	8.3	55	13.1	18	9.9	19	12.8	73	12.1	1.000	92	12.3
≥1 p.I155T[a]	0	0	0	0	2	3.7	1	3.6	-	-	0	0	8	1.9	1	0.5	3	2.0	9	1.5	1.000	12	1.6
≥1 p.R160W[a]	0	0	3	9.1	14	25.9	4	14.3	-	-	5	20.8	32	7.6	13	7.1	26	17.4	45	7.5	**<0.005**	71	9.5
≥1 p.R163Q	0	0	0	0	6	11.1	13	46.4	-	-	2	8.3	25	6	6	3.3	21	14.1	31	5.2	**<0.005**	52	6.9
≥1 p.D294H[a]	1	10	1	3.0	3	5.6	1	3.6	-	-	2	8.3	63	15	17	9.3	8	5.4	80	13.3	**0.045**	88	11.7

[a]R variants: variants highly associated with the red hair color phenotype

P-values were obtained comparing Latin America and Spain. Adjusted p-values were calculated using Bonferroni correction.

Table S2. Clinical and phenotypic characteristics of melanoma patients with at least 1 *MC1R* variant by country

	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		**LATIN AMERICA**			**SPAIN**			**TOTAL**		
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Adj. P	Nº	%	Adj. P	Nº	%	Adj. P
Hair color																									
red	1	100	5	100	2	100	0	0	-	-	4	100	22	100	13	86.7	12	92.3		35	94.6		47	94.0	
blond	2	100	12	70.6	29	82.6	7	87.5	-	-	2	100	58	71.6	27	60.0	42	80.8	1.000	79	68.1	**<0.002**	121	72.0	**<0.002**
dark	6	85.7	6	54.5	24	82.8	15	78.9	-	-	11	78.6	159	68.2	62	57.9	62	77.5		221	65.0		283	67.4	
missing	1		0		0		0		-	-	4		69		11		4			73			77		
TOTAL	9		23		45		22		-	-	21		301		107		120			408			528		
Eye color																									
fair	5	100	13	68.4	20	83.3	10	71.4	-	-	8	88.9	92	69.7	32	68.1	56	78.9	1.000	124	69.3	1.000	180	72.0	1.000
dark	4	80.0	10	71.4	25	83.3	12	85.7	-	-	9	81.8	144	72.0	66	57.9	60	81.1		210	66.9		270	69.6	
missing	0		0		0		0		-	-	4		65		9		4			74			78		
TOTAL	9		23		45		22		-	-	21		301		107		120			408			528		
Skin color[a]																									
fair	6	85.7	22	68.8	42	89.4	17	81.0	-	-	16	94.1	144	75.8	47	67.1	103	83.1	0.185	191	73.5	0.070	294	76.6	**<0.002**
dark	3	100	1	100	3	12.9	5	71.4	-	-	1	33.3	108	68.4	53	54.6	13	61.9		161	63.1		174	63.0	
missing	0		0		0		0		-	-	4		49		7		4			56			60		
TOTAL	9		23		45		22		-	-	21		301		107		120			408			528		
Nevi count																									
<50	-	-	10	90.9	28	80.0	6	66.7	-	-	9	81.8	84	69.4	42	60.9	53	80.3		126	66.3		179	69.9	
50-100	-	-	2	66.7	9	100	7	77.8	-	-	3	100	55	72.4	15	75.0	21	87.5	1.000	70	72.9	1.000	91	75.8	1.000
>100	-	-	5	62.5	8	80.0	9	90.0	-	-	4	100	97	70.8	4	80.0	26	81.3		101	71.1		127	73.0	
missing	-	-	6		0		0		-	-	5		65		46		4			111			131		
TOTAL	-	-	23		45		22		-	-	21		301		107		120			408			528		
Nº MM																									
1	5	100	10	66.7	17	77.3	11	78.6	-	-	1	100	131	72.8	65	52.0	44	77.2		196	64.3		240	66.3	
2	3	75.0	4	57.1	18	85.7	8	72.7	-	-	6	100	125	72.7	33	71.7	39	79.6	1.000	158	72.5	0.950	197	73.8	0.130
3	1	100	5	71.4	6	100	3	100	-	-	0	0	28	65.1	5	71.4	15	88.2		33	66.0		48	71.6	
≥4	0	0	4	100	4	80	0	0	-	-	3	100	15	71.4	4	100	11	91.7		19	76.0		30	81.1	
missing	0		0		0		0		-	-	11		2		0		11			2			13		
TOTAL	9		23		49		22		-	-	21		301		107		120			408			528		
	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		**LATIN AMERICA**			**SPAIN**			**TOTAL**		
Age at diagnosis of first melanoma[b]	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	p-value	y.o.	SD	p-value	y.o.	SD	p-value
MC1R WT	58.0	-	53.7	12.2	50.9	9.9	55.8	16.7	-	-	49.0	1.4	45.6	16.0	47.7	16.4	53.0	11.4		46.4	16.2		47.2	15.8	
Al least 1 MC1R variant	42.8	12.7	52.2	14.6	45.4	12.7	47.7	14.4	-	-	43.8	19.1	45.2	16.2	47.2	16.8	46.7	14.6	**0.040**	45.8	16.3	0.652	46.0	16.0	0.335
TOTAL	44.3	12.9	52.7	13.7	46.3	12.4	49.2	14.8	-	-	44.3	18.1	45.3	16.1	47.4	16.6	47.9	14.2		46.0	16.3		46.4	15.9	

Nº MM=total primary melanomas, y.o.=years. [a]Skin color was classified according to the phototype Fitzpatrick classification being fair (phototypes I or II) and dark (phototypes III to V). [b] The age at diagnosis of first melanoma was not available in 1/28 (3.4%) of Chilean, 2/20 (10%) of Uruguayan, 64/707 (9.1%) of Barcelonan and 3/195 (1.5%) of Valencian patients.*P*-values were obtained comparing carriers vs. non-carriers individually in Latin America, Spain or in the Total set of samples. Adjusted *P* were calculated using the Bonferroni correction.

Table S3. Clinical and phenotypic characteristics of melanoma patients with at least 1 RHC variant by country

	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		**LATIN AMERICA**			**SPAIN**			**TOTAL**		
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	Adj. *P*	*N*	%	Adj. *P*	*N*	%	Adj. *P*
Hair color																									
red	1	100	2	40.0	1	50.0	0	0	-	-	4	100	19	86.4	13	86.7	8	61.5		32	86.5		40	80.0	
blond	1	50.0	7	41.2	16	69.6	2	25.0	-	-	2	100	31	38.3	12	34.3	28	53.8	**0.005**	43	37.1	**<0.002**	71	42.3	**<0.002**
dark	2	28.6	1	9.1	10	34.5	5	26.3	-	-	3	21.4	85	36.5	21	19.6	22	26.3		106	31.2		127	30.2	
missing	0		0		0		0		-	-	2		33		4		2			37			39		
TOTAL	4		10		27		7		-	-	11		168		50		59			218			277		
Eye color																									
fair	2	40.0	6	31.6	14	58.3	3	21.4	-	-	3	33.3	53	40.2	16	34.0	28	39.4		69	38.5		97	38.8	
dark	2	40.0	4	28.6	13	48.1	4	28.6	-	-	6	54.5	79	39.5	31	27.2	29	39.2	1.000	110	35.0	1.000	139	35.8	1.000
missing	0		0		0		0		-	-	2		36		3		2			39			41		
TOTAL	4		10		27		7		-	-	11		168		50		59			218			277		
Skin color[a]																									
fair	3	72.9	10	31.3	27	57.4	5	23.8	-	-	9	52.9	76	40.0	29	41.4	54	43.5		105	40.4		159	41.4	
dark	1	33.3	0	0	0	0	2	28.6	-	-	0	0	63	39.9	17	17.5	3	14.3	0.070	80	31.4	0.175	83	30.1	**0.015**
missing	0		0		0		0		-	-	2		29		4		2			33			35		
TOTAL	4		10		27		7		-	-	11		168		50		59			218			277		
Nevi count																									
<50	-	-	4	36.4	18	51.4	1	11.1	-	-	4	36.4	48	39.7	18	26.1	27	40.9		66	34.7		93	36.3	
50-100	-	-	0	0	4	44.4	1	11.1	-	-	1	33.3	28	36.8	8	40.0	6	25.0	1.000	36	37.5	1.000	42	35.0	1.000
>100	-	-	2	25.0	5	50.0	5	50.0	-	-	3	75.0	56	40.9	2	40.0	15	46.9		58	40.8		73	42.0	
missing	-	-	4		0		0		-	-	3		36		22		11			58			69		
TOTAL	-	-	10		27		7		-	-	11		168		50		62			218			277		
Nº MM																									
1	3	60.0	4	26.7	13	59.1	4	28.6	-	-	1	100	73	40.6	37	29.6	25	43.9		110	36.1		135	37.3	
2	1	25.0	2	28.6	10	47.6	3	27.3	-	-	2	33.3	67	39.0	11	23.9	18	36.7		78	35.8		96	36.0	
3	0	0	2	28.6	4	66.7	0	0	-	-	0	0	14	32.6	1	14.3	6	35.3	1.000	15	30.0	1.000	21	31.3	1.000
≥4	0	0	2	50.0	0	0	0	0	-	-	1	33.3	12	57.1	1	25.0	3	25.0		13	52.0		16	43.2	
missing	0		0		0		0		-	-	7		2		0		7			2			9		
TOTAL	4		10		27		7		-	-	11		168		50		61			218			277		

	Argentina		Brazil (Porto Alegre)		Brazil (Sao Paulo)		Chile		Mexico		Uruguay		Spain (Barcelona)		Spain (Valencia)		**LATIN AMERICA**			**SPAIN**			**TOTAL**		
Age at diagnosis of first melanoma[b]	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	y.o.	SD	p-value	y.o.	SD	p-value	y.o.	SD	p-value
RHC variants	44.7	16.2	53.4	15.6	45.4	11.7	40.1	12.1	-	-	48.8	17.6	45.3	16.2	45.6	16.9	46.6	13.8		45.4	16.3		45.6	15.8	
no-RHC variants	44.0	11.9	52.4	13.2	47.2	13.2	52.4	14.6	-	-	40.3	18.6	45.3	16.1	48.1	16.5	48.8	14.4	0.383	46.3	16.3	0.508	46.8	16.0	0.360
TOTAL	44.3	12.9	52.7	13.7	46.3	12.4	49.2	14.8	-	-	44.3	18.1	45.3	16.1	47.4	16.6	47.9	14.2		46.0	16.3		46.4	15.9	

N MM=total primary melanomas.[a]Skin color was classified according to the phototype Fitzpatrick classification being fair (phototypes I or II) and dark (phototypes III to V). [b]The age at diagnosis of first melanoma was not available in 1/28 (3.4%) of Chilean, 2/20 (10%) of Uruguayan, 64/707 (9.1%) of Barcelonan and 3/195 (1.5%) of Valencian patients. *P*-values were obtained comparing carriers vs. non-carriers individually in Latin America, Spain or in the Total set of samples. Adjusted *P* were calculated using the Bonferroni correction.

Annex 3

Additional Article 3

Title: Genetic susceptibility to cutaneous melanoma in southern Switzerland: role of *CDKN2A, MC1R* and *MITF*.

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Publication: Br J Dermatol. 2016;175(5):1030-1037. Doi: 10.1111/bjd.14897.

Impact Factor (JCR Science Edition 2016): 4.706 (1st decile, DERMATOLOGY)

Contribution of the doctoral student to the article: The doctoral student participated in genetic analysis and microsatellite genotyping of the patients, performed part of the statistical data analysis and participated writing part of the original draft of the manuscript.

GENERAL DERMATOLOGY

BJD
British Journal of Dermatology

Genetic susceptibility to cutaneous melanoma in southern Switzerland: role of *CDKN2A*, *MC1R* and *MITF*

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Summary

Background Nearly 10% of all cases of cutaneous melanoma (CM) occur in patients with a personal or family history of the disease.

Objectives To obtain information about genetic predisposition to CM in Ticino, the southern region of Switzerland, a zone with moderate-to-high CM incidence.

Methods We identified germline mutations in highly CM-associated genes (*CDKN2A* and *CDK4*) and low/medium-penetrance variants (*MC1R* and *MITF*) in patients with multiple primary CMs or individuals with one or more CM and a positive family history for CM or pancreatic cancer among first- or second-degree relatives. Healthy blood donors (n = 146) were included as a control group.

Results From July 2010 to July 2012, 57 patients (41 pedigrees) were included. Twenty-six were melanoma-prone families (with at least two cases) and 15 had multiple CMs. Pancreatic cancer was found in six families. The CDKN2A mutation p.V126D was identified in seven patients (four families) with a founder effect, whereas CDKN2A A148T was detected in seven cases (five families) and seven healthy donors (odds ratio 2·76, 95% confidence interval 0·83–9·20). At least one MC1R melanoma-associated polymorphism was detected in 32 patients (78%) and 97 healthy donors (66%), with more than one polymorphism in 12 patients (29%) and 25 healthy donors (17%). The MITF variant p.E318K was identified in four patients from three additional pedigrees (7%) and one healthy control (0·7%).

Conclusions Inclusion criteria for the Ticino population for genetic assessment should follow the rule of two (two affected individuals in a family or a patient with multiple CMs), as we detected a CDKN2A mutation in almost 10% of our pedigrees (four of 41), MITF p.E318K in 7% (three of 41) and a higher number of MC1R variants than in the control population.

What's already known about this topic?

- *CDKN2A* as a high-penetrance risk factor and MITF and MCR1, with low-to-intermediate penetrance, are the most important genes involved in melanoma susceptibility.
- Mutation detection rates in these genes are highly variable across regions.

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Accepted for publication
21 July 2016

Funding sources
This study was supported by scientific funding from the dermatology department of the Ospedale Regionale Bellinzona e Valli. The research at the Melanoma Unit at Hospital Clinic Barcelona is funded by grants from Fondo de Investigaciones Sanitarias P.I. 09/01393 and P.I. 12/00840, Spain; by the CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain, cofunded by 'Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una Manera de Hacer Europa'; and by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain.

Conflicts of interest
None declared.

DOI 10.1111/bjd.14897

- *CDKN2A* mutation detection increases with the number of melanomas, young age at diagnosis and concomitant pancreatic cancer in the family.

What does this study add?

- The genetic predisposition to melanoma in southern Switzerland is analysed for the first time.
- A CDKN2A high-risk mutation is detected in almost 10% of pedigrees and MITF p.E318K mutation in 7%.
- It is difficult to establish a rule for recommending genetic testing based on only the number of melanomas in the family or the individual.

Susceptibility to cutaneous melanoma (CM) is a heterogeneous field, with interaction between external (environmental) and internal (genetic) factors. Most CMs are sporadic, but 5–10% can be considered familial as they occur in patients with a previous family history of the disease.[1] Among genes involved in CM susceptibility with high penetrance, the *CDKN2A* locus on chromosome 9p21 is the most important and frequently mutated. This gene encodes two distinct proteins, both with a tumour suppressor function: p16INK4A and p14ARF.[1–4]

Germline mutations in *CDKN2A* have been described in an average of 20–40% of hereditary cases of melanoma.[1,5] A review of the literature on studies of genetic testing in melanoma published from 1994 to 2007 shows that the likelihood of *CDKN2A* mutation detection increases with the number of melanomas in the family, the combination of both pancreatic cancer and CM in the same family, younger onset of CM diagnosis and the number of primary CM diagnoses in one individual, in spite of a lack of familial predisposition.[1,5,6] The second CM susceptibility gene, *CDK4*, is an oncogene located on 12q13. Worldwide, few cases of *CDK4*-mutated families have been published.[7–9]

Recently, genes involved in telomere function (*ACD*, *TERF2IP*, *POT1*) have also been implicated in melanomagenesis.[6] Some publications have identified loss-of-function variants in the protection of telomeres 1 gene (*POT1*) in several populations from Europe, the U.S.A. and Australia, suggesting that *POT1* is another major susceptibility gene for familial CM.[10–12] Regarding intermediate- or low-penetrance genes, the melanocortin receptor 1 gene (*MC1R*) is one of the most studied. *MC1R* (located in 16q24) encodes the melanocyte-stimulating hormone receptor, which is implicated in the pigmentation process. The *MC1R* gene is highly polymorphic in humans, with more than 100 variants reported, the majority being nonsynonymous.

Some of these *MC1R* variants are associated with red hair phenotype, poor tanning ability and freckles, and they are named red hair colour (RHC) variants, in contrast to variants not associated with red hair, named not red hair colour (NRHC) variants.[4,13,14] Recently, it has been demonstrated that mainly RHC variants but also some NRHC variants (called 'r' alleles, rHC) increase CM risk in families with or without known *CDKN2A* mutations.[13,15] Moreover, some studies have demonstrated a correlation between the number of RHC and rHC variants and the risk of CM.[4,6,14]

Recently, some studies have implicated the BRCA1-associated protein 1 gene (*BAP1*) and the microphthalmia-associated transcription factor gene (*MITF*) as high- and intermediate-penetrance melanoma predisposition genes, respectively.[16–19] Germline *BAP1* mutations are associated with a novel cancer syndrome characterized by an increased risk of malignant mesothelioma, atypical melanocytic tumours (melanocytic BAP1-mutated atypical intradermal tumours), uveal and cutaneous melanoma and other neoplasms.[17] The MITF mutation p.E318K is associated with both familial and sporadic melanoma susceptibility and/or renal cell carcinoma risk, an increased naevus count and nonblue eye colour.[6,16,18,19]

In spite of these important and recent studies on new genes implicated in melanoma susceptibility, genetic counselling is still only broadly recommended for high-risk melanoma gene (*CDKN2A* and *CDK4*) mutation carriers, and it should be performed in genetic referral units and/or as a part of a research investigation programme.[1]

The incidence of CM in southern Switzerland is considered moderate to high with around 20–25 cases per 100 000 habitants.[20,21] Analysis on genetic predisposition to CM has never been performed in this area, located between the northern and southern Alps. The purpose of this study was to evaluate the genetic predisposition to CM in individuals with a family or personal history of CM in southern Switzerland.

Patients and methods

This was a cohort study designed and developed by the dermatology department of Ente Ospedaliero Cantonale, Bellinzona, Switzerland, and the melanoma unit of Hospital Clinic Barcelona, IDIBAPS, Spain. The aim was to identify germline mutations in CM-associated genes (*CDKN2A*, *CDK4*, *MC1R* and *MITF*) in patients with at least two CMs in the individual (at least one of them invasive; in spite of the absence of a family history of CM) or in the family, or at least one CM and one or more pancreatic cancer among first- or second-degree

relatives on the same side of the family. In the case that a high-risk germline mutation was found in any of the affected individuals, the possibility of participating in the study was offered to the rest of the family. Eligible patients were recruited with the collaboration of private practice dermatologists from southern Switzerland and oncologists from Istituto Oncologico della Svizzera Italiana. Diagnosis of CM was confirmed by review of histological materials, pathology reports and/or medical records.

Moreover, we included a set of 146 healthy blood donors as a control group from the same geographical area. All of the controls had no familial or personal history of melanoma or other cancers. The same demographic and phenotypic data as for the patients were recorded.

Patients' samples were encoded to process data anonymously. Information about genetic counselling, psychological support, appropriate screening and education was offered to all of the families and individuals regardless of the results of genetic testing.

The study was approved by the ethical committees of both centres, and informed consent was obtained from all individuals.

Data collection

Demographic characteristics (date of birth and sex), skin phenotype (hair and eye colour, freckles, skin phototype, naevus count, atypical naevi, histologically proven dysplastic naevi), clinical data (age at diagnosis of CM, location of CM, number of primary CMs, personal history of other cancers) and familial data (familial relationships, number of familial CMs, family history of other cancers) were collected for each individual. *CDKN2A* and *CDK4* mutation status (no mutation/homozygous wild-type vs. presence of mutation/variation) and location of the mutation were also collected. *MC1R* genotypes were classified as RHC- or rHC-associated risk variants, or wild-type, or synonymous variant for the rest of the variants as previously described.[22,23] The *MITF* variant encoding the p.E318K mutant (rs149617956) was also genotyped.

Genetic testing

During visit 1, genomic DNA was obtained from 2 mL of saliva. DNA was extracted using the commercial Oragene DNA Collection Kit following the manufacturer's instructions (DNA Genotek, Ottawa, ON, Canada). *CDKN2A* exons 1α, 1β and 3, and exon 2 of *CDK4* were amplified by polymerase chain reaction (PCR) using the primers previously described.[3] Exon 2 of *CDKN2A* was amplified using primers forward 5′-CTGG-CTCTGACCATTCTGTT-3′ and reverse 5′-CTCTGAGCTTTG-GAAGCTCTC-3′. Direct sequencing was used as the mutation screening technique. *MC1R* was directly sequenced,[24] MITF variant p.E318K (rs149617956) was analysed using the Custom TaqMan® SNP Genotyping Assays as previously described,[16] and plates were read on a 7900HT Fast Real-Time PCR System with Sequence Detection Software (all Applied Biosystems, Foster City, CA, U.S.A.).

Six markers were used for haplotype analysis along the 9p21 locus: D9S736, D9S1749, D9S942, D9S1748, D9S171 and D9S126. Primer sequences were obtained from the Genome Database. PCR assays were performed using fluorescently labelled primers and run in an ABI3130 sequencer (Applied Biosystems). Complete details of the PCR conditions are available on request. Allele sizes for all markers are comparable with those from previous haplotype studies[25–27] (except for D9S736 and D9S126).

Statistical analysis

SPSS 20·0 (IBM, Armonk, NY, U.S.A.) was used to analyse the data. All analyses were tested at the 0·05 significance level. To evaluate the association between categorical variables, Pearson's χ^2 or Fischer's exact tests were used (last test for frequency classes lower than five or when the total number of observations was < 20). To compare the means of quantitative variables, Student's t-test or ANOVA were used as applicable. Melanoma index case (first patient with melanoma studied) probands were used for the statistical analysis.

Results

Clinical data

Fifty-seven individuals were included in the study: 41 unrelated patients with melanoma, seven relatives with melanoma and nine relatives without melanoma. The cases of melanoma consisted of 15 patients with multiple CMs without family history and 33 cases in 26 melanoma-prone families (two or more cases of melanoma in the same family): one family with six affected members, seven families with three and 18 families with two. Regarding other cancers, we found nine patients with melanoma with a personal history of other cancers (three patients with nonmelanoma skin cancer, two with thyroid cancer, two with gynaecological cancer, one with gastrointestinal cancer and one with central nervous system cancer). We have observed only four pedigrees with no other member affected by cancer, and 21 pedigrees with more than one member affected. Pancreatic cancer was detected in six pedigrees and breast cancer in eight.

Index patients with melanoma (15 patients with multiple CMs without family history and the index case of each of the 26 melanoma-prone families, n = 41) and the control population were compared. This showed that patients had a statistically significantly greater presence of a risk phenotype, along with atypical and dysplastic naevi, freckles, less tanning ability and lighter eye and hair colour (Table 1).

CDKN2A/*CDK4* mutations

Seven patients, belonging to four different melanoma-prone families, were carriers of the same c.377T>A (p.V126D) mutation in *CDKN2A* (Table S1; see Supporting Information). Haplotype analysis of these families suggested a common

Table 1 Genetic and phenotypic characteristics in proband patients with melanoma (n = 41) and a control population (n = 146) from Ticino, Switzerland

	Patients, n (%)	Controls, n (%)	OR	95% CI	P-value
CDKN2A					
p.A148T (polymorphism)	5 (12)	7 (4·8)	2·76	0·83–9·20	0·14
p.V126D (mutation)	4 (10)	0	–	–	0·002
MITF					
p.E318K	3 (7)	1 (0·7)	11·37	1·15–112·4	0·034
MC1R variants[a]					
0	9 (22)	49 (33·6)	0·56	0·24–1·26	0·15
1	20 (49)	72 (49·3)	0·98	0·49–1·96	
2	12 (29)	25 (17·1)	2·00	0·90–4·45	
≥ 1 variants (all)	32 (78)	97 (66·4)	1·80	0·80–4·06	0·18
≥ 1 red hair colour variants	16 (39)	39 (26·7)	1·75	0·85–3·63	0·17
Naevus count > 50	31 (76)	42 (29·4)	7·45	3·35–16·57	< 0·001
Atypical naevi present	31 (76)	10 (9·3)	30·38	11·57–79·75	< 0·001
Dysplastic naevi present	25 (61)	19 (13·1)	10·36	4·70–22·87	< 0·001
Freckles: some to many	36 (88)	30 (20·4)	28·08	10·15–77·70	< 0·001
Tanning ability: burn to little tan	26 (63)	34 (23·1)	5·76	2·74–12·10	< 0·001
Eye colour light	32 (78)	74 (50·3)	3·51	1·56–7·86	0·002
Hair colour					
Red	10 (24)	1 (0·7)	–	–	< 0·001
Blonde	15 (37)	24 (16·3)			
Dark	16 (39)	122 (83·0)			

OR, odds ratio; CI, confidence interval. [a]Synonymous variants were excluded from all of the analyses.

ancestral origin for this mutation in at least three of them (Fig. 1). Overall, 25% of the families with at least three cases of CM, 11% of families with two cases (two of 18) and 6% of multiple cases of CM without a family history (one of 15) were *CDKN2A* mutation carriers. No *CDKN2A* mutation was identified in cancer-free controls from Ticino (Table 2; see Supporting Information).

The presence of the CDKN2A mutation was associated with early age at diagnosis in all cases of melanoma (n = 48) (P = 0·036) (Table 2). All patients with melanoma carrying this mutation had atypical naevi and > 50 common naevi. Family history of pancreatic cancer was found in two of four (50%) families with mutation in *CDKN2A*, and four of 37 (11%) families without mutations (Table 3). There was not a significantly increased risk for pancreatic cancer in individuals carriers of the V126D mutation (odds ratio 8·25, 95% confidence interval 0·91–75·79).

No mutation was detected in exon 2 of *CDK4*.

CDKN2A variants

We identified seven patients with melanoma (from five different families, three of them belonging to the same family) who were carriers of variant c.442G>A (p.A148T) in *CDKN2A*. This variant was more frequent in melanoma index cases than in the control population, but it did not reach significance (12% vs. 4·8%, P = 0·14) (Table 1). We also observed three uncommon variants: c.−33G>C in individual TI031-01; c.−45G>A in individual TI00102; and c.273G>A (p.L91L) in individuals TI040-01 and TI040-03. p.L91L also affects transcript p14ARF (p.G106R, c.316G>A). It has been detected only once in a European non-Finnish population, with a frequency of $1{\cdot}67 \times 10^{-5}$ and 59 928 alleles studied (ExAC database, http://exac.broadinstitute.org/), and rarely in African populations (frequency $8{\cdot}52 \times 10^{-4}$). This variant did not segregate with melanoma in the family.

Frequency of *MC1R* variants

At least one MC1R variant (RHC: c.86dupA, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H and/or rHC: p.V60L, p.V92M, p.R163Q and other missense rare variants not listed as RHC) was identified in 78% of index patients with melanoma, with more than one *MC1R* variant in 29% of patients. Although RHC variants were more frequent in index patients (39%) than in the southern Switzerland control population (26·7%), this did not reach statistical significance due to the size effect. We have not detected any statistical differences in the number of variants (0, 1 or ≥ 2, P = 0·15) between patients with melanoma and the control population (Table 1). The presence of any *MC1R* variants (RHC or rHC) was associated with having > 50 naevi in patients with melanoma (P = 0·025) and a higher number of freckles in the control population (P < 0·001) (Table 4 and Table S3; see Supporting Information). Age at CM diagnosis was not different depending on the number of *MC1R* variants (RHC or rHC) or the presence or absence of RHC variants (Table 2).

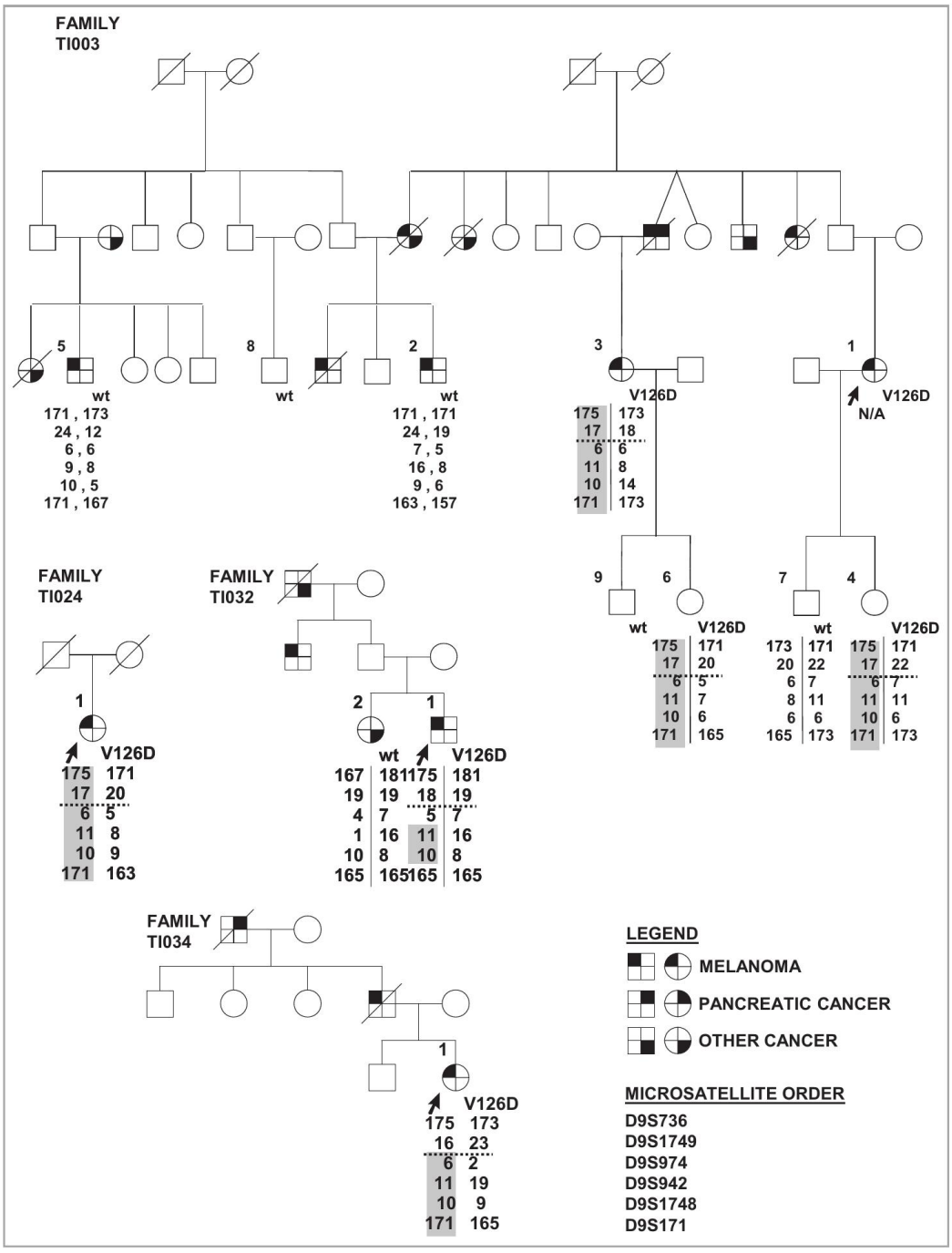

Fig 1. Pedigrees with mutation p.V126D in CDKN2A. Index cases are indicated with an arrow. CDKN2 status is indicated as wt (wild-type) or V126D (when the individual carries the p.V126D mutation). Genotypes for six markers at the 9p21 locus are depicted below each individual. Haplotype cosegregation with the mutation in family TI003 and the common haplotype within the other families with the mutation are indicated by shadowed boxes. The dotted line indicates the genomic location of the p.V126D mutation. For families TI024 and TI034 the haplotype shown is the most probable, but could not be confirmed by analysing other relatives.

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Table 2 Mean age at diagnosis in all patients with melanoma (n = 48) according to genetic features

	Age at diagnosis of first melanoma (years), mean ± SD
0 MC1R variants	37·1 ± 14·9
1 MC1R variant (RHC or rHC)[a]	43·0 ± 16·0
≥ 2 MC1R variants (RHC or rHC)[a]	47·0 ± 13·3
P-value	0·29
Presence of RHC	44·6 ± 15·1
Absence of RHC	42·4 ± 15·5
P-value	0·64
Presence of p.V126D in CDKN2A	35·4 ± 6·2
Absence of p.V126D in CDKN2A	44·1 ± 15·5
P-value	0·036
Total	43·2 ± 15·0

[a]RHC (red hair colour): c.86dupA, p.R142H, p.R151C, p.I155T, p.R160W and p.D294H; rHC (not red hair colour): p.V60L, p.V92M, p.R163Q and other missense rare variants not listed as RHC.

Table 3 Frequency of nonmelanoma skin cancers in the families and mutation status for the *CDKN2A* and *MITF* genes

Family history of cancer type	*MITF* mutation	*CDKN2A* mutation	No known risk mutation	Total
Pancreas	0	2	4	6
Breast	1	1	6	8
Laryngeal	0	1	4	5
Genitourinary (e.g. prostate, bladder)	0	0	8	8
Gastrointestinal tract	0	1	11	12
Lung	1	1	5	7
Nervous system	0	2	1	3
Thyroid	0	1	1	2
Uterus	2	0	4	6
Haematological (e.g. leukaemia, lymphoma)	2	1	1	4
Kidney	0	0	1	1
Others (e.g. nonmelanoma skin cancer, liver, unknown)	0	0	6	6

MITF p.E318K variant

The MITF p.E318K variant was detected in three index patients with melanoma (TI012-01, TI014-01 and TI039-01) belonging to three different families (two families and one patient with multiple melanoma). This variant segregates with the disease in family TI014 (TI014-02 was also positive) but not in any other family (Table S1; see Supporting Information). We did not find any statistically significant association between the presence of variant p.E318K and clinical or phenotypical characteristics. p.E318K was detected in one patient from our control population (frequency 0·7%) and was related to melanoma susceptibility in Ticino (odds ratio 11·37, 95% confidence interval 1·15–112·4; P = 0·034) (Table 1).

Discussion

The purpose of this study was to observe the implication of high- and medium-susceptibility genes in melanoma predisposition in a cohort of patients with a family or personal history of CM in southern Switzerland, an area with moderate-to-high melanoma incidence.

Leachman *et al.*[1] proposed that only individuals with ≥ 10% probability of carrying a mutation should be considered as candidates for genetic testing. In the same review the authors described a very useful rule for testing depending on melanoma incidence in the studied area.[1,6] As suggested in the literature, the inclusion criteria for genetic testing in our population should be those of a high- or moderate-incidence area of melanoma. The criteria would be (i) the presence of at least three cases of CM in the same individual or family and/or (ii) the concomitance of at least two CMs and/or pancreatic cancer. If we had followed these criteria, we would not have detected one mutation-carrying family with only two cases of melanoma (TI032) carrying the p.V126D mutation in CDKN2A. Moreover, three more cases, in which the medium-risk susceptibility variant p.E318K in MITF was detected, would not have been identified.

Taking into account our results, and in spite of having a moderate-to-high incidence of CM in this area, the rule of two could be followed for genetic testing, as a CDKN2A high-risk mutation is detected in 11·5% of families and 6·6% of patients with multiple primary melanomas.

Interestingly, the four families were carriers of the same missense mutation (p.V126D). This was previously identified as a high-risk melanoma mutation in Italian, French and American populations.[5,6,24] As we expected, haplotype analysis suggested a common origin for at least three of them. As in the families presented by Goldstein *et al.*,[26] one family had the 5 allele at D9S974, rather than the 6 allele seen in the remaining families. The authors could not disclose whether this two-base-pair difference could be due to a recombination event or replication slippage. As marker D9S974 is the closest to the mutation, if a recombination was the case it would indicate a remote origin of the mutation, while replication slippage would not indicate so.

The MITF p.E318K variant was identified in three additional cases (two families and one patient). The occurrence (7%) of this variant among families is higher than reported previously for other populations.[16,18,19] Also, contrary to what has been reported, no kidney cancer was detected in these three families. This fact must highlight the necessity of individualized studies in any community before counselling is offered in a clinical setting, as other authors recommend.[1,2]

Moreover, as we have discussed above, it is difficult to establish a rule for recommending genetic testing based only

Table 4 Clinical and phenotypic characteristics in proband patients with melanoma (n = 41) and the control population (n = 146) from Ticino, Switzerland, according to MC1R variants

	Patients: MC1R ≥ 1 variants (all)[a]					Controls: MC1R ≥ 1 variants (all)[a]				
	Yes, n (%)	No, n (%)	OR	95% CI	P-value	Yes, n (%)	No, n (%)	OR	95% CI	P-value
Naevus count > 50	27 (84)	4 (44)	6·75	1·33–34·27	0·025	25 (28)	16 (33)	0·76	0·36–1·61	0·56
Atypical naevi present	26 (81)	5 (56)	3·47	0·71–16·94	0·19	5 (7)	5 (14)	0·44	0·12–1·64	0·29
Dysplastic naevi present	19 (59)	6 (67)	0·73	0·15–3·46	1·00	11 (12)	7 (14)	0·79	0·28–2·17	0·79
Freckles: some to many	30 (94)	6 (67)	7·50	1·02–55·00	0·061	27 (28)	2 (4)	9·06	2·06–39·94	< 0·001
Tanning ability: burn to little tan	21 (66)	5 (56)	1·53	0·34–6·87	0·70	25 (26)	8 (16)	1·78	0·74–4·31	0·22
Eye colour light	26 (81)	6 (67)	2·17	0·42–11·24	0·38	50 (52)	24 (49)	1·11	0·56–2·20	0·86
Hair colour										
Red	6 (19)	0	–	–	0·35	1 (1)	0	–	–	0·57
Blonde	15 (47)	4 (44)				19 (20)	6 (12)			
Dark	11 (34)	5 (56)				77 (79)	43 (88)			

OR, odds ratio; CI, confidence interval. [a]Synonymous variants were excluded from all the analyses. The following are included: RHC (red hair colour): c.86dupA, p.R142H, p.R151C, p.I155T, p.R160W and p.D294H; rHC (not red hair colour): p.V60L, p.V92M, p.R163Q and other missense rare variants not listed as RHC.

on the number of CMs in the family or the individual. Perhaps other factors such as phenotype, history of other cancers in the family or the genetic characteristics from the particular geographical area studied can play an important role in the genetic predisposition to CM and the probability of finding a mutation in known predisposition genes. In our case, it must be emphasized that only five patients did not report other cancer-affected relatives. Interestingly, there were important percentages of families with a history of pancreatic, breast or gastrointestinal cancer (15%, 20% and 32%, respectively).

It is remarkable that 50% (two of four) of the *CDKN2A* mutation carrier families had one member affected by pancreatic cancer. At the moment, our data do not clarify the causes of additional cancer risk in these melanoma-prone families, but demonstrate the importance of integrating family history and risk assessment into clinical practice to identify patients who may be at increased risk of cancer and who can benefit from a complete cancer genetics programme.[1,5]

Another crucial issue when genetic counselling is offered is the importance of the psychological aspects and potential changing of preventive behaviour in the families or individuals studied.[28] We have certainly considered these aspects in our series but the data are not presented in this paper (ongoing analysis).

We detected the p.A148T variant in CDKN2A in seven patients, belonging to five families (12%). This variant is controversial. It has been implicated in melanoma susceptibility in some studies[3,5,29] but not in others.[29,30] Taking as a reference other control populations, geographically close to ours, this percentage remains high (6·2% in the Italian population, 4·2% in the French population).[30,31] In our series, this variant seems to be more frequent among patients with CM than in healthy donors, but the difference is not statistically significant.

Regarding *MC1R* variants, we have detected an important percentage of index cases (78%) with one or more RHC or rHC variant. These *MC1R* variants also increase melanoma risk not only in *CDKN2A*-positive mutation carriers,[13] but also in *CDKN2A* nonmutation carriers.[10,15] Interestingly, the presence of *MC1R* variants, besides being associated with the presence of freckles, as previously reported,[32] was associated with having a high naevus count in patients with melanoma. There is one article suggesting that *MC1R* could have a role in naevus growth,[33] but no other studies have reported this association.

In conclusion, we report the genetic results for melanoma predisposition in selected patients from southern Switzerland. In this cohort, we identified four families carrying a high-risk mutation (p.V126D) in CDKN2A and sharing a common ancestral origin, and an additional three cases with the rare MITF variant, p.E318K. We also detected an important number of patients with low-risk variants (RHC and/or rHC) in the *MC1R* gene. Our results may explain, in part, the moderate-to-high incidence of melanoma in this population.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Patients' and their relatives' genetic results.

Table S2. Control genetic results.

Table S3. Clinical and phenotypic characteristics in proband patients with melanoma (n = 41) and the control population (n = 146) from Ticino (Switzerland) according to MC1R red hair colour variants (c.86dupA, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H).

Supplementary Table 1. Patients and relatives genetic results

DNA	**Sex**	**Age at First MM**	**Number of primary MM**	***CDKN2A***	***MITF***	***MC1R****
TI001-01	M	45	1	WT	WT	p.V92M, p.R151C
TI001-02	M	25	2	WT	WT	p.V92M
TI002-01	F	15	2	WT	WT	WT
TI003-01	F	34	4	**p.V126D**	WT	c.86dupA
TI003-02	M	62	1	WT	WT	p.V60L, p.V92M
TI003-03	F	38	1	**p.V126D**	WT	p.V60L (HOM)
TI003-04	F		0	**p.V126D**	WT	p.R142H
TI003-05	M	49	1	WT	WT	c.86dupA, p.V92M
TI003-06	F		0	**p.V126D**	WT	p.V60L
TI003-07	M		0	WT	WT	p.V60L
TI-003-08	M		0	WT	WT	p.V92M
TI-003-09	F		0	WT	WT	p.V60L, p.V92M
TI004-01	M	58	2	WT	WT	p.V60L
TI005-01	F	38	2	WT	WT	p.V60L
TI006-01	M	62	2	WT	WT	p.V60L, p.V92M
TI007-01	F	39	1	WT	WT	p.V60L (HOM)
TI-008-01	M	34	3	WT	WT	p.R160W
TI-009-01	F	50	2	WT	WT	WT
TI-010-01	M	39	1	WT	WT	p.R163Q
TI-011-01	F	32	2	**p.A148T**	WT	p.D294H
TI-012-01	M	47	1	**p.A148T (HOM)**	**p.E318K**	WT
TI-012-02	F	21	1	**p.A148T**	WT	p.V60L

DNA	**Sex**	**Age at First MM**	**Number of primary MM**	***CDKN2A***	***MITF***	***MC1R****
TI-012-03	F		0	**p.A148T**	WT	p.V60L
TI-013-01	F	42	1	WT	WT	p.V92M, p.R160W
TI-014-01	M	37	2	WT	**p.E318K**	p.V60L, p.R160W
TI-014-02	F	55	1	WT	**p.E318K**	p.R160W
TI-014-03	M		0	WT	WT	WT
TI-015-01	F	42	2	**p.A148T**	WT	p.R142H
TI-015-02	M		0	WT	WT	p.R142H
TI-016-01	F	30	1	WT	WT	WT
TI-017-01	M	76	3	WT	WT	p.R160W
TI-018-01	F	36	3	WT	WT	p.V60L
TI-019-01	F	46	1	WT	WT	p.V60L
TI-020-01	M	59	2	WT	WT	p.R151C, p.R160W, p.R229H
TI-021-01	F	24	2	WT	WT	p.D84E, p.V92M
TI-022-01	F	38	1	WT	WT	p.V92M
TI-023-01	M	29	1	WT	WT	p.V60L
TI-024-01	F	44	11	**p.V126D**	WT	c.86dupA (Hom)
TI-025-01	F	18	2	WT	WT	WT
TI-026-01	M	49	2	WT	WT	WT
TI-027-01	F	45	2	WT	WT	p.V60L
TI-028-01	M	73	2	WT	WT	p.V92M
TI-029-01	M	67	2	WT	WT	p.V60L (HOM)
TI-030-01	F	34	3	WT	WT	WT
TI-031-01	F	69	2	WT	WT	p.R142H

DNA	**Sex**	**Age at First MM**	**Number of primary MM**	***CDKN2A***	***MITF***	***MC1R****
TI-032-01	M	34	1	**p.V126D, p.A148T**	WT	p.R160W
TI-032-02	F		0	**p.A148T**	WT	WT
TI-033-01	F	58	3	WT	WT	WT
TI-034-01	F	27	2	**p.V126D**	WT	p.D294H
TI-035-01	F	32	2	WT	WT	p.R151C, p.V208I
TI-036-01	M	40	1	WT	WT	p.V60L
TI-037-01	F	52	1	WT	WT	p.V60L, p.V92M
TI-038-01	F	33	1	WT	WT	WT
TI-039-01	F	29	2	WT	**p.E318K**	p.V92M
TI-040-01	M	68	1	**p.L91L (p.G106R in p14ARF), p.A148T**	WT	p.V92M
TI-040-02	M	33	2	**p.A148T (HOM)**	WT	p.V60L, p.V92M
TI-040-03	M		0	**p.L91L (p.G106R in p14ARF)**	WT	p.V60L
TI-041-01	M	67	1	WT	WT	p.V60L, p.R151C

All variants and mutations listed were found in heterozygosis except those marked (HOM), which were found in homozygosis. WT: wild type

*Only non-synonymous variants are listed

CDKN2A mutations are expressed according to p16INK4A protein change except for those indicated within parentheses.

Supplementary Table 2. Control genetic results

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 0001	M	48	WT	WT	p.D294H
TIC 0002	M	47	WT	WT	p.V60L
TIC 0003	M	40	WT	WT	p.V60L
TIC 0004	F	42	WT	WT	p.D294H
TIC 0005	M	65	NA	WT	p.R160W
TIC 0006	M	50	WT	WT	p.V60L (HOM)
TIC 0007	F	56	WT	WT	WT
TIC 0008	M	26	WT	WT	p.V92M, p.R151C
TIC 0009	M	41	WT	WT	p.R151C
TIC 0010	M	48	WT	WT	WT
TIC 0011	M	46	WT	WT	p.P256S
TIC 0012	F	50	WT	WT	WT
TIC 0013	M	53	WT	WT	p.R142H
TIC 0014	M	55	WT	WT	WT
TIC 0015	M	55	WT	WT	WT
TIC 0016	M	45	WT	WT	p.R151C
TIC 0017	M	49	WT	WT	p.R160W
TIC 0018	M	71	WT	WT	p.D294H
TIC 0019	M	47	WT	WT	WT
TIC 0020	F	34	WT	WT	p.V60L
TIC 0021	M	56	**p.A148T**	WT	c.86insA, p.V60L
TIC 0022	M	49	WT	WT	p.R163Q
TIC 0023	M	52	WT	WT	WT
TIC 0024	M	21	WT	WT	p.V60L (HOM)

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 0025	F	61	WT	WT	p.R163Q, p.D294H
TIC 0026	M	49	WT	WT	WT
TIC 0027	M	52	WT	WT	WT
TIC 0028	M	45	WT	WT	p.V92M, p.R151C
TIC 0029	F	21	WT	WT	p.I155T
TIC 0030	F	24	WT	WT	WT
TIC 0031	M	42	WT	WT	WT
TIC 0032	M	38	**p.A148T**	WT	WT
TIC 0033	M	25	WT	WT	WT
TIC 0034	M	37	WT	WT	WT
TIC 0035	M	45	WT	WT	WT
TIC 0036	M	28	WT	WT	WT
TIC 0037	M	23	WT	WT	p.V60L, p.I155T
TIC 0038	M	58	WT	WT	p.V60L, p.V92M
TIC 0039	M	51	WT	WT	WT
TIC 0040	F	61	WT	WT	p.V60L, p.V92M
TIC 0041	M	63	WT	WT	WT
TIC 0042	F	56	WT	WT	p.V92M
TIC 0043	F	65	WT	WT	p.V60L
TIC 0044	F	30	WT	WT	p.V60L
TIC 0045	F	21	WT	WT	WT
TIC 0046	F	20	WT	WT	p.V60L
TIC 0047	F	21	WT	WT	p.V92M
TIC 0048	F	24	WT	WT	p.R151H
TIC 0049	F	59	WT	WT	p.V92M

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 0050	M	33	WT	WT	WT
TIC 0051	M	52	WT	WT	p.R160W
TIC 0052	F	23	WT	WT	WT
TIC 0053	F	31	WT	**p.E318K**	p.R160W
TIC 0054	F	29	WT	WT	WT
TIC 0055	F	36	WT	WT	WT
TIC 0056	M	59	WT	WT	p.V60L
TIC 0057	M	59	WT	WT	WT
TIC 0058	M	63	WT	WT	p.V60L
TIC 0059	M	68	WT	WT	p.R160W
TIC 0060	F	38	WT	WT	WT
TIC 0061	M	47	WT	WT	WT
TIC 0062	M	46	WT	WT	p.V92M
TIC 0063	M	24	WT	WT	p.V92M
TIC 0064	M	51	WT	WT	p.R160W
TIC 0065	F	39	WT	WT	p.V60L (HOM)
TIC 0066	M	57	WT	WT	p.V92M
TIC 0067	F	31	WT	WT	p.V60L
TIC 0068	F	28	**p.A148T**	WT	p.V60L
TIC 0069	M	28	WT	WT	p.V92M
TIC 0070	F	57	WT	WT	p.R160W
TIC 0071	F	57	**p.A148T**	WT	WT
TIC 0072	M	57	WT	WT	WT
TIC 0073	F	50	WT	WT	p.V60L (HOM)
TIC 0074	M	37	WT	WT	WT

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 0075	M	56	WT	WT	p.V92M
TIC 0076	F	51	WT	WT	p.R160W
TIC 0077	M	53	WT	WT	WT
TIC 0078	F	58	WT	WT	WT
TIC 0079	F	52	WT	WT	p.R163Q (HOM)
TIC 0080	M	44	WT	WT	WT
TIC 0081	M	56	WT	WT	p.V92M, p. R142H
TIC 0082	F	28	WT	WT	p.V92M
TIC 0083	F	55	WT	WT	p.R160W
TIC 0084	F	52	WT	WT	p.V92M (HOM)
TIC 0085	M	23	WT	WT	p.D294H
TIC 0086	M	23	WT	WT	p.V92M, p.R160W
TIC 0087	M	43	WT	WT	p.R142H
TIC 0088	M	62	WT	WT	p.V60L, p.D294H
TIC 0089	M	62	WT	WT	p.V92M
TIC 0090	M	50	WT	WT	p.V92M
TIC 0091	F	50	WT	WT	p.D294H
TIC 0092	M	54	WT	WT	p.V60L, p.R160W
TIC 0093	M	21	WT	WT	WT
TIC 0094	M	29	WT	WT	WT
TIC 0095	M	49	WT	WT	p.V60L
TIC 0096	M	28	WT	WT	p.R160W
TIC 0097	M	40	WT	WT	WT
TIC 0098	M	32	**p.A148T**	WT	p.R160W
TIC 0099	M	28	WT	WT	p.R160W

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 0100	M	57	WT	WT	p.R160W
TIC 0101	F	30	WT	WT	WT
TIC 0102	F	50	WT	WT	WT
TIC 0103	F	60	WT	WT	p.V92M
TIC 0104	F	64	WT	WT	p.V60L
TIC 0105	F	59	WT	WT	p.V60L (HOM)
TIC 0106	M	59	WT	WT	p.V60L, p.R163Q
TIC 0107	M	48	WT	WT	p.V60L
TIC 0108	M	52	WT	WT	p.R163Q
TIC 0109	M	48	WT	WT	WT
TIC 0110	M	68	WT	WT	p.V92M, p.R163Q
TIC 0111	F	60	WT	WT	p.V60L
TIC 0112	F	73	WT	WT	WT
TIC 0113	M	23	WT	WT	p.V60L
TIC 0114	F	48	WT	WT	p.R151C
TIC 0115	M	46	WT	WT	p.V60L
TIC 0116	M	46	WT	WT	p.V92M
TIC 0117	M	52	WT	WT	p.V60L
TIC 00118	F	50	WT	WT	WT
TIC 00119	F	54	WT	WT	WT
TIC 00120	M	23	WT	WT	p.V60L
TIC 00121	M	22	WT	WT	p.V60L (HOM)
TIC 00122	M	23	WT	WT	p.R163Q
TIC 00123	F	26	WT	WT	p.R151C
TIC 00124	F	22	WT	WT	WT

ID	**Sex**	**Age**	***CDKN2A***	***MITF***	***MC1R****
TIC 00125	F	43	WT	WT	p.R151C
TIC 00126	M	37	WT	WT	p.V92M
TIC 00127	M	49	WT	WT	p.V60L, p.V92M
TIC 00128	M	54	WT	WT	WT
TIC 00129	F	48	WT	WT	p.D294H
TIC 00130	M	58	WT	WT	WT
TIC 00131	M	41	WT	WT	WT
TIC 00132	M	55	WT	WT	p.D294H
TIC 00133	M	36	WT	WT	p.R151C
TIC 00134	M	47	WT	WT	WT
TIC 00135	M	69	**p.A148T**	WT	WT
TIC 00136	F	50	WT	WT	p.V92M, p.R163Q
TIC 00137	M	58	WT	WT	p.V92M, p.T95M
TIC 00138	M	43	WT	WT	p.V60L
TIC 00139	M	44	NA	NA	NA
TIC 00140	F	38	WT	WT	p.V60L
TIC 00141	M	40	WT	WT	WT
TIC 00142	F	22	**p.A148T**	NA	p.V60L
TIC 00143	M	59	WT	WT	p.V92M
TIC 00144	M	29	WT	WT	p.R151C
TIC 00145	F	41	WT	WT	WT
TIC 00146	M	21	WT	WT	p.V92M
TIC 00147	F	34	WT	WT	p.V60L (HOM)

All variants and mutations listed were found in heterozygosis except those marked (HOM), which were found in homozygosis. WT: wild type

*Only non-synonymous variants are listed

Supplementary Table 3. Clinical and phenotypic characteristics in proband melanoma patients (n=41) and control population (n=146) from Ticino (Switzerland) according to *MC1R* RHC variants (c.86dupA, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H)

	PATIENTS							CONTROLS						
	MC1R□□□□RHC variants							MC1R□□□□RHC variants						
	Yes		No					Yes		No				
	N	%	N	%	OR	95% CI	*P*	N	%	N	%	OR	95% CI	*P*
Nevi count														
>50	13	81.3	18	72.0	1.69	0.37-7.78	0.712	10	27.8	31	29.2	1.08	0.46-2.49	1.000
Atypical nevi														
Presence	13	81.3	18	72.0	1.69	0.37-7.78	0.712	1	3.6	9	11.2	0.29	0.04-2.42	0.448
Dysplastic nevi														
Presence	10	62.5	15	60.0	1.11	0.31-4.04	1.000	7	17.9	11	10.5	1.86	0.67-5.23	0.260
Freckles														
Some-Many	15	93.8	21	84.0	2.86	0.29-28.20	0.632	12	30.8	17	15.9	2.35	1 00-5.53	0.061
Tanning ability														
Burn-Little Tan	9	56.3	17	68.0	0.61	0.17-2.21	0.517	13	33.3	20	18.7	2.17	0.95-4.96	0.075
Eye colour														
Light	11	68.8	21	84.0	0.42	0.09-1.88	0.276	23	59.0	51	47.7	1.57	0.75-3.31	0.264
Hair colour														
Red	5	31.2	1	4.0				1	2.6	0	0.0			
Blonde	5	31.2	14	56.0	-	-	0.048	9	23.1	16	15.0	-	-	0.094
Dark	6	37.5	10	40.0				29	74.5	91	85.0			

Annex 4

Low-risk melanoma genes and their function

Gene	Gene Name	Function	Reference
AGR3	Anterior gradient 3, protein disulphide isomerase family member	Generates and modifies disulphide bonds during protein folding	58
ARNT	Aryl hydrocarbon receptor nuclear translocator	xenobiotic metabolism, regulates CYP1B1	58
ASIP	Agouti signaling protein	antagonist of α-MSH	58
ATM	ATM serine/threonine kinase	cell cycle checkpoint kinase that phosphorylates, among others, DNA repair genes	58
CASP8	Caspase 8	apoptosis	58
CCND1	Ciclin D1	regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition	58
CDKAL1	CDK5 regulatory subunit associated protein 1 like 1	modulates gene expression, including proinsulin, via tRNA methylthiolatio	58
CYP1B1	cytochrome P450 family 1 subfamily B member 1	Lipid, hormone and exogenous chemicals metabolism	58
FTO	Fat mass and obesity associated	protein related with the non-heme iron enzymes	65
GSTP1	Glutathione S-transferase pi 1	xenobiotic metabolism, ROS detoxification	64
HLA class II	major histocompatibility complex /Human leukocyte	Antigen presenting molecules	62

Gene	Gene Name	Function	Reference
genes	antigen		
IL10	Interleukin 10	immunoregulation and inflammation	57
IL1B	Interleukin 1 beta	mediator of the inflammatory response	57
IRF4	Interferon Regulatory Factor 4	Nevi count, melanocyte and lymphocyte regulator	63
MTAP	Methylthioadenosine phosphorylase	human pigmentation, associated with nevi count	60
MX2	MX dynamin like GTPase 2	Function not clearly understood. May be involved in immune system.	58
OCA2	Oculocutaneous albinism II	eye color determination and pigmentation	59
PARP1	Poly ADP-Ribose Polymerase 1	chromatin-associated enzyme that modifies nuclear proteins, involved in DNA repair	63
PAX3	Paired box 3	face and eye development, associated with nevi count	61
PLA2G6	Phospholipase A2 group VI	Lipid metabolism, associated with nevi count	58
RAD23B	RAD23 homolog B, nucleotide excision repair protein	nucleotide excision repair protein	58
SLC45A2	Solute carrier family 45 member 2	Skin and hair pigmentation	58
STN1 (*OBFC1*)	STN1, CST complex subunit	component of the telomere maintenance complex	58

Gene	Gene Name	Function	Reference
TERT	Telomerase reverse transcriptase	maintains telomere ends by addition of the telomere repeat TTAGGG, associated with nevi count	58
TNFα	Tumor necrosis factor alpha	multifunctional proinflammatory cytokine	57
TYR	Tyrosinase	eye color determination and tanning ability	58
TYRP1	Tyrosinase-related protein 1	stabilizes TYR, involved in pigmentation	58
VDR	Vitamin D receptor	mineral metabolism, associated with nevi count	66

Annex 5

Staging classification table adapted from the Melanoma AJCC Staging 8th Edition (2017) [81]

Stage	T	Thickness (mm)	Ulceration status	N	No. tumor-involved regional lymph nodes	Presence of in-transit, satellite, and/or microsatellite metastases	And m is…	Anatomic site
0	Tis	Not applicable		N0	No regional metastases detected	No	M0	No evidence of distant metastasis
IA	T1a	<0.8	Without ulceration					
IA	T1b	<0.8	With ulceration					
IA	T1b	0.8–1.0	Any					
IB	T2a	>1.0–2.0	Without ulceration					
IIA	T2b	>1.0–2.0	With ulceration					
IIA	T3a	>2.0–4.0	Without ulceration					
IIB	T3b	>2.0–4.0	With ulceration					
IIB	T4a	>4.0	Without ulceration					
IIC	T4b	>4.0	With ulceration					
IIIA	T1a/b–T2a	see above	see above	N1a	One clinically occult	No		
				N2a	Two or 3 clinically occult	No		
IIIB	T1a/b–T2a	see above	see above	N1b	One clinically detected	No		
				N1c	No regional lymph node disease	Yes		
				N2b	Two or 3, at least one of which was clinically detected	No		
IIIB	T2b/T3a	see above	see above	N1a–N2b	see above	see above		
IIIB	T0	No evidence of primary tumor		N1b, N1c	see above	see above		
IIIC	T0	No evidence of primary tumor		N2b	see above	see above		
				N2c	One clinically occult or clinically detected	Yes		

Stage	T	Thickness (mm)	Ulceration status	N	No. tumor-involved regional lymph nodes	Presence of in-transit, satellite, and/or microsatellite metastases	And m is…	Anatomic site
							M0	No evidence of distant metastasis
IIIC	T1a–T3a	see above	see above	N3a	Four or more clinically occult	No		
IIIC	T0	No evidence of primary tumor		N3b	Four or more, at least one of which was clinically detected, or the presence of any number of matted nodes	No		
				N3c	Two or more clinically occult or clinically detected and/or presence of any number of matted nodes	Yes		
IIIC	T1a–T3a	see above	see above	N2c, N3b, N3c	see above	see above		
IIIC	T3b/T4a	see above	see above	Any N ≥N1	at least one			
IIIC	T4b	see above	see above	N1a–N2c	see above	see above		
IIID	T4b	see above	see above	N3a/b/c	see above	see above		
IV	Any	Any	Any	Any	Any	Any	M1	Distant metastasis
IVA							M1a	Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph node
IVB							M1b	Distant metastasis to lung with or without M1a sites of disease
IVC							M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b

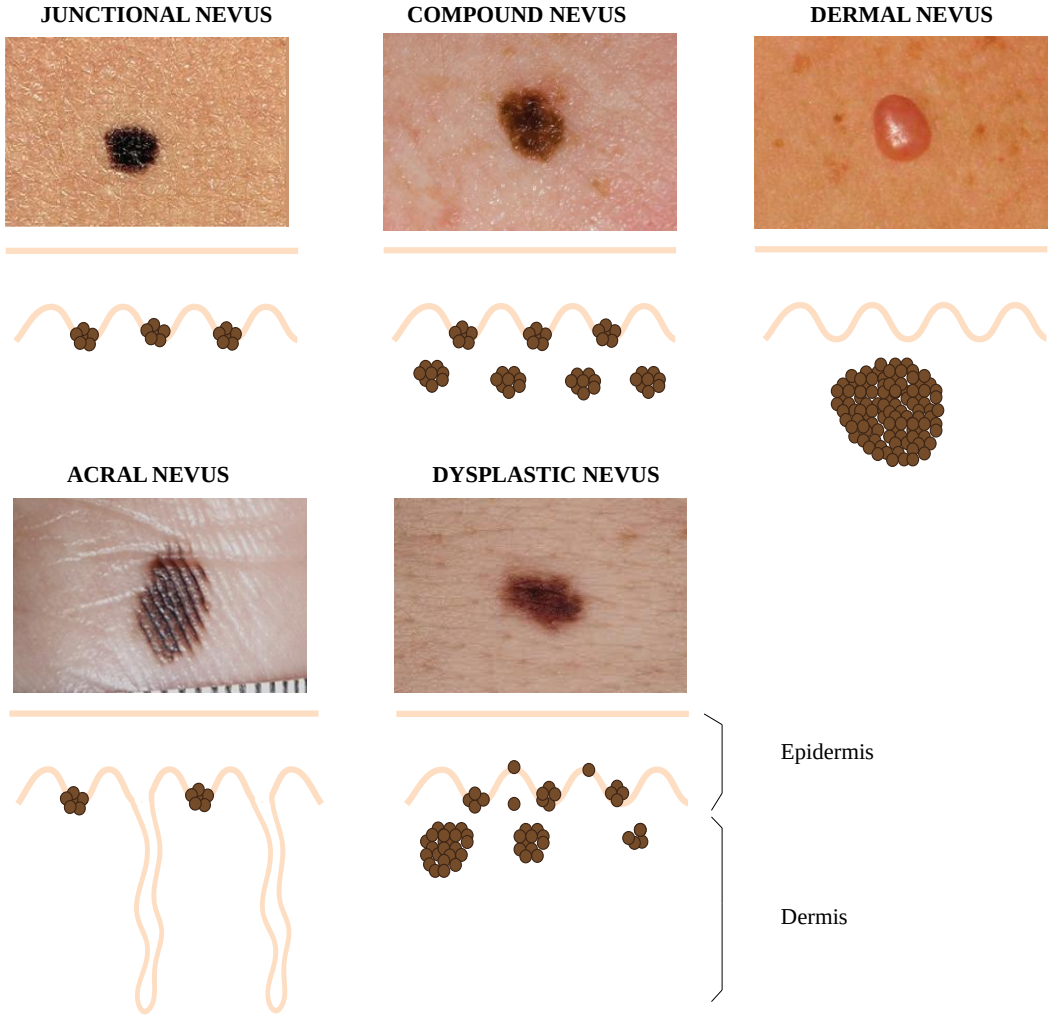
Stage	T	Thickness (mm)	Ulceration status	N	No. tumor-involved regional lymph nodes	Presence of in-transit, satellite, and/or microsatellite metastases	And m is…	Anatomic site
	Any	Any	Any	Any	Any	Any		sites of disease
IVD							M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease

CNS indicates central nervous system; LDH, lactate dehydrogenase.

In M1, if LDH is recorded a suffix can be used: (0) LDH not elevated, (1) LDH elevated.

Annex 6

Nevi types



Clinical image example for each of five nevi type is shown. Below each clinical image, a schema of a skin section with the most representative traits for each type is drawn. Brown round cells represent nevocytes.

Annex 7

Current distribution of prevalent pathogenic variants according to gene affected in MPM and familial melanoma in Barcelona according to the number of primaries/cases

MPM: sporadic multiple primary melanoma patients; WT: wild-type

Each central round represents high-risk genes, the medial circle represents *MITF* status and the external circle represents *MC1R* status. *MITF* and *MC1R* data was not available for a subset of patients (19% and 36%, respectively, in MPM and 50% and 28%, respectively, in families). For the graphical representation, the variant percentage has been extrapolated for *MITF* and *MC1R* missing data, considering that variant proportion will be maintained in each group with the increase of sample size.

For melanoma-prone families, only index cases were considered.

For sporadic MPM and melanoma-prone families visited at the Melanoma Unit of Hospital Clínic of Barcelona data derived from articles 1, 2 and 3, Annex 2 and additional sequenced individuals during the last two years in our Unit, including also a family carrying a *BAP1* mutation (data not published).